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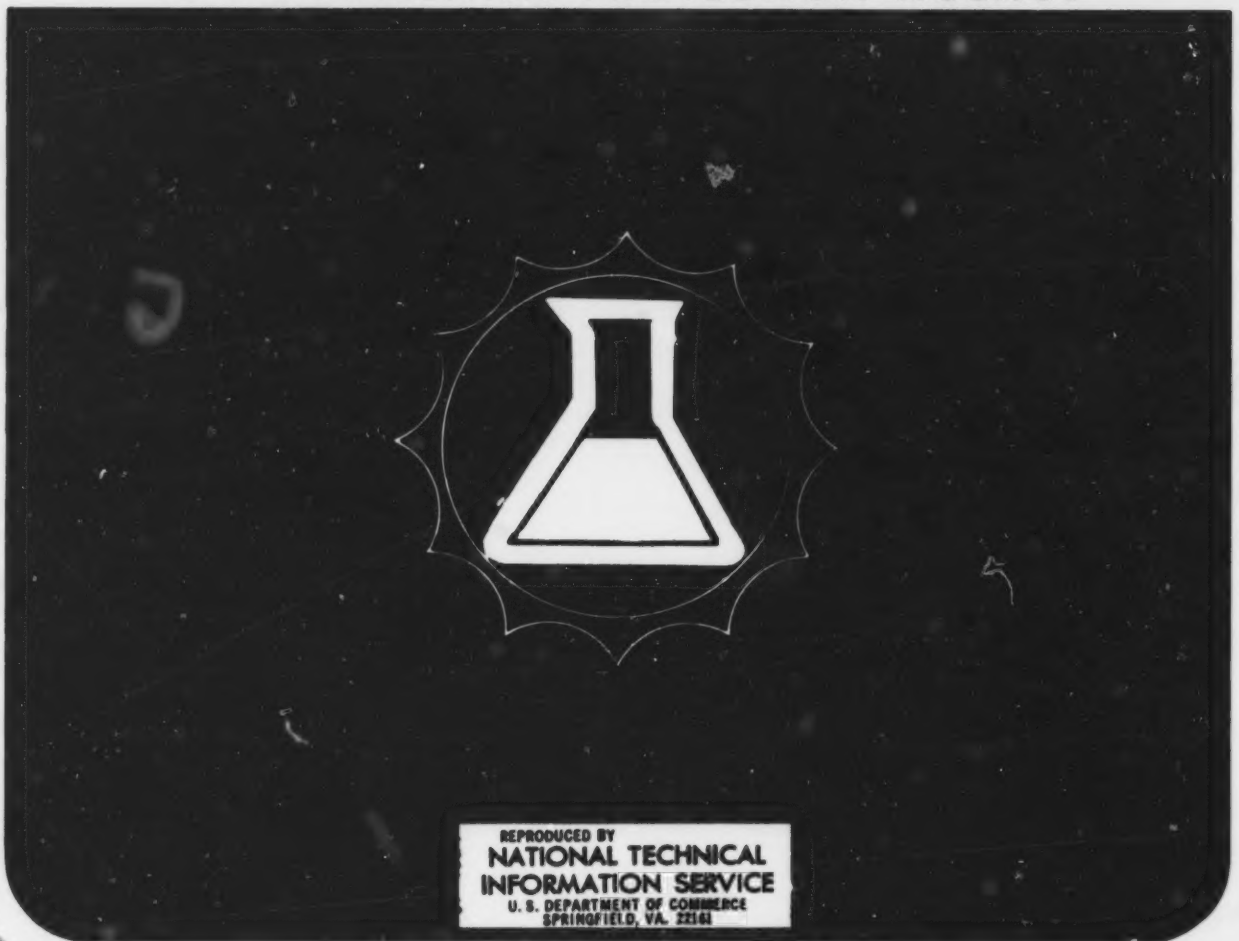
Algal Assay Procedure
Bottle Test

NATIONAL EUTROPHICATION RESEARCH PROGRAM

ENVIRONMENTAL PROTECTION AGENCY



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ALGAL ASSAY PROCEDURE

BOTTLE TEST

National Eutrophication Research Program
Environmental Protection Agency

August 1971

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FOREWORD

This document is the product of more then two years of intensive research at governmental, industrial, and university laboratories to develop a reliable and reproducible algal assay. The "Bottle Test" of the Provisional Algal Assay Procedure (PAAP), which was published by the Joint Industry/Government Task Force on Eutrophication in February, 1969, served as the starting point for this effort. In improving and evaluating the test, each laboratory, of the participating group of nine, followed the same research plan using algal test species from a common source. This course of action identified elements of the test that were faulty or difficult or questionable. As such specific problems were recognized, selected laboratories were assigned the task of probing them and developing sound adjustments. To do this they followed research plans developed jointly by all laboratories.

As a result of this massive effort, the Algal Assay Procedure: Bottle Test has been refined sufficiently to be offered now for wider use in connection with eutrophication and other algal production problems. This point in progress has been attained only through the intense interest and continuing energies of the participating laboratories. These laboratories and the personnel who worked on this project are shown on the following page.

Coordination of this program has been the responsibility of Mr. Thomas E. Maloney of the National Eutrophication Research Program, Pacific Northwest Water Laboratory, Corvallis, Oregon. To him goes much credit for the effective way in which the program has moved continuously and effectively toward this goal.

Finally, the way is now open to move on with the next step--that of learning to use this newly improved procedure as an aid in solving practical problems. This publication is one step in stimulating such action.

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ALGAL ASSAY PROCEDURE: BOTTLE TEST

1. Introduction

With the surge of interest in the growing problem of eutrophication, the Joint Industry/Government Task Force on Eutrophication recognized that acceptable standardized algal growth tests must be developed as a tool in controlling eutrophication. While many investigators have improvised algal assays to meet their specific needs, these assays offer no basis for comparison of acceptably reproducible results between laboratories or on samples obtained from different geographic areas. In February 1969 the Joint Task Force published the Provisional Algal Assay Procedure (PAAP) The PAAP was developed from the collective knowledge and experience of persons who had fundamental knowledge of algal physiology, algal growth responses, and experience with algal assays of various types. It was determined that the PAAP should consist of three fundamental test procedures: a Bottle Test, a Continuous-Flow Chemostat Test, and an *In situ* Test. It was fully recognized that the PAAP was tentative and that a great deal of research would be necessary to sharpen each of the three procedures to determine their capabilities and to compare test performance one with the other.

Shortly after publication of the PAAP, a group consisting of government, university and industrial laboratories undertook a comprehensive research program to improve and evaluate it. The program was coordinated by the National Eutrophication Research Program of the Federal Water Quality Administration located at the Pacific Northwest Water Laboratory, Corvallis, Oregon.

The first phase was concerned with comparing the Bottle Test and "Continuous-Flow" Chemostat Test for assaying the algal growth-nutrient concentration relationships in natural and enriched waters. Each laboratory followed the same research plan for the evaluation using algal test species from a common source. When specific problems were recognized, certain laboratories were assigned to investigate them after all laboratories had agreed to the research plan to be followed. In interlaboratory precision tests, using the Bottle Test, excellent agreement in the data was obtained by the participating laboratories. After nearly two years of research it is now felt that the Bottle Test had undergone sufficient evaluation and refinement to be considered reliable. As a result this document, the Algal Assay Procedure: Bottle Test, was developed.

Publication of this document does not imply that the Continuous-Flow Chemostat Test or the In situ Test are no longer a part of the PAAP. Both are considered important procedures, but further research must be conducted in order to evaluate and refine them before they become ready for universal use. Neither should it be implied that, because the Bottle Test has been extensively tested, future changes or additions will not be incorporated into it. Just as with all procedures, it will very likely be updated from time to time. Although the laboratory procedure itself is ready for routine use, further evaluation relating to several specific field situations will be necessary. Also, this procedure will serve as a basis of comparison for further evaluation of the Continuous-Flow Chemostat and In situ tests.

2. Principle

This algal assay is based on Liebig's law of the minimum which states that "growth is limited by the substance that is present in minimal quantity in respect to the needs of the organism." The test in its present form is intended primarily for use in the following general situations.

1. Assessment of a receiving water to determine its nutrient status and sensitivity to change.

2. Evaluation of materials and products to determine their potential effects on algal growth in receiving waters.

3. Assessment of effects of changes in waste treatment processes on receiving waters.

Bottle algal assays consist of three steps, (1) selection and measurement of appropriate parameters during the assay (for example, biomass indicators such as total cell carbon), (2) presentation and statistical evaluation of the measurements made during the assay, and (3) interpretation of the results with respect to the specific problem being investigated. Specifically, it is intended that the test be used: (1) to identify algal growth-limiting nutrients; (2) to determine biologically the availability of algal growth-limiting nutrients; and (3) to quantify the biological response to changes in concentrations of algal growth-limiting nutrients. These measurements are made by adding a selected test alga to the test water and determining algal growth at appropriate intervals.

The test may also be used to determine whether or not various compounds or water samples are toxic or inhibitory to algae. In this connection caution should be observed when interpreting results when there is little or no growth response in samples where sufficient nutrients appear to be or are, in fact, present. The presence of toxicants can prevent or inhibit algal growth even when nutrients are not limiting.

It should be pointed out that test flasks are normally incubated to facilitate free gas exchange at the air-water interface. Therefore, since atmospheric carbon dioxide is available, the test as outlined cannot be used to demonstrate algal growth limitations due to lack of carbon in the water. The test, however, can be modified to obtain such information, but more research is necessary.

2.1 Maximum specific growth rate is related to the concentration of the rate limiting nutrient present. Maximum standing crop is proportional to the initial amount of the limiting nutrient available.

2.2 Growth response - All comparative growth responses should be analyzed statistically and significant levels of the differences reported. For most purposes a 95 percent significance level can be considered statistically significant.

3. Planning and Evaluation of Algal Assays

The specific experimental design of each algal assay must be dictated by the actual situation. It is extremely important that all pertinent ecological factors be considered in the planning of a given assay to insure that valid results and conclusions are

obtained. As a minimum, the following specifics must be considered by each investigator who plans to conduct algal assays for the purposes listed above.

3.1 Selection of test waters - Water quality may vary greatly with time and with location in lakes, impoundments and streams. Sampling programs should be established so that meaningful data will be obtained.

3.11 Spatial variations - In a stratified lake or impoundment it may be of value to sample both epilimnetic and hypolimnetic waters. The use of transection lines are helpful in sampling; samples from a transection can be taken from major depth zones. In rivers and streams useful information may be obtained by taking samples upstream and downstream from suspected nutrient sources and from tributary streams. When materials are evaluated, samples from a number of natural waters having a range of representative water qualities must be included.

3.12 Temporal variations - The nutrient content of natural and waste waters often vary greatly with time. The variation may not only be seasonal, but hourly. When sampling, this must be taken into consideration.

3.2 Determination of limiting nutrients - Any of the essential nutrients may limit algal growth. Bioassays can be designed to examine in detail only a few nutrients which by preliminary testing have been shown to be most likely limiting or in short supply. An example of an experimental plan is given in Appendix 3.2.

3.3 Evaluation of materials - In planning a program for evaluating a material for its potential effect on receiving waters the following factors must be considered:

(1) The distribution of the test material; e.g., local, regional, national.

(2) The level of test material to be used; e.g., usage in product or other measure of material involved.

(3) The chemical and/or physical nature of the material; e.g., its theoretical potential for direct nutrient enrichment or of indirect effects.

(4) The fate of the material; e.g., its chemical change or biological degradation in waste treatment or surface waters.

(5) The pathway by which the material will reach the receiving water; e.g., consideration of including the test material in an appropriate range of waste treatment effluents or as a component of surface runoff.

(6) The dilution factor for the stream receiving wastes containing the test material; e.g., an appropriate range of dilutions for introduction to receiving waters.

(7) The selection of appropriate test water; e.g., use of appropriate test water for a material of local interest, but use of full range of water qualities for material with broad distribution.

An example of an experimental plan is given in Appendix 3.3.

3.4 Assessment of waste treatment processes - The algal assay may be used to determine the algal growth stimulatory effects of a given process effluent. When the assay is used for this purpose, the overall evaluation must include consideration of the following aspects:

(1) Typical and atypical conditions, nationwide and/or local, under which the type of process effluent may enter the environment.

(2) Growth parameter(s) and test organism(s) to be used.

(3) Which nutrient(s) is (are) limiting growth?

(4) Is there a change in the growth limiting nutrient as a result of the process?

(5) What is the overall effect of a process or process change?

An example of one possible treatment evaluation is shown in Appendix 3.4.

4. Apparatus

4.1 Sampling and sample preparation.

4.11 Water sampler - non-metallic.

4.12 Sample bottles - autoclavable (such as borosilicate glass, linear polyethylene, polycarbonate, or polypropylene).

4.13 Membrane filter apparatus - for use with 47 mm pre-filter pads and an 0.45μ porosity filter.

4.14 Autoclave or pressure cooker - capable of producing 15 psi (1.1 kg/cm^2) at 250° F (121° C).

4.2 Culturing and incubation.

4.21 Culture vessels - Erlenmeyer flasks of good quality borosilicate glass such as Pyrex or Kimax. The same brand of glass should be used within laboratory. When trace nutrients are being studied special glassware, such as Vycor, polycarbonate, or coated glassware should be used.

While the flask size is not critical, due to carbon dioxide limitation, the surface to volume ratios are. The recommended surface to volume ratios are as follows:

40 ml of sample in 125 ml flask

60 ml of sample in 250 ml flask

100 ml of sample in 500 ml flask

It is desirable to number permanently test flasks in order that anomalous growth which appears to be related to specific flasks can be identified and those flasks eliminated from future tests.

4.22 Culture closures - foam plugs, loose fitting aluminum foil or inverted beakers must be used to permit good gas exchange (see Sec 9.2) and prevent contamination. Each laboratory must determine for each batch of closures purchased whether that batch has any significant effect on the maximum specific growth rate and/or the maximum standing crop.

4.23 Constant temperature room, or equivalent incubator, capable of providing temperature control at 24 $\pm$ 2° C.

4.24 Illumination - "Cool-White" fluorescent lighting to provide 400 ft-c (4304 lux) $\pm$10 percent or 200 ft-c (2152 lux) $\pm$10 percent measured adjacent to the flask at the liquid level.

4.25 Light meter - several types may be used, but must be calibrated against a standard light source or light meter (see Appendix 8).

4.3 General

4.31 Balance, analytical, capable of weighing 100 gm with a precision of ± 0.1 mg.

4.32 Microscope - good quality general purpose.

4.33 Microscope illuminator - good quality general purpose.

4.34 Hemacytometer or plankton counting slide.

4.35 pH meter - scale of 0-14 pH units with accuracy of ± 0.1 pH unit.

4.36 Oven, dry heat capable of temperatures of 120° C.

4.37 Centrifuge - capable of relative centrifugal force of at least 1,000 x g.

4.38 Spectrophotometer or colorimeter - for use at 600-750mμ

4.4 Optional

4.41 Electronic cell counter.

4.42 Fluorometer.

4.43 Shaker table, capable of 100 oscillations per minute.

5. Sample Collection, Transport, Preparation and Storage

5.1 Collection - Use non-metallic water sampler and autoclavable storage containers (see Sections 4.11 and 4.12). Containers should not be re-used when toxic or nutrient contamination is suspected.

5.2 Transport conditions - Leave a minimum of air space in transport container, keep in dark and at ice temperature.

5.3 Preparation - To enable the use of unialgal test species the indigenous algae must be "removed" before assaying the sample.

This "removal" necessitates either the separation or destruction of the indigenous algae in the sample. Filtration and autoclaving are recommended and the use of one or both depends upon the type of information being sought. The effects of some pretreatment methods are shown in Appendix 5.3.

5.31 Membrane filtration - should be used when it is desired to remove indigenous algae to determine growth-limiting soluble nutrients, which have not been taken up by filterable organisms, or in order to predict the effect of adding nutrients to a test water at a specific time. Pretreat 0.45μ porosity membrane filter by passing at least 50 ml of distilled water through it. Discard filtrate. Then filter quantity of the sample as needed under reduced pressure of 0.5 atmosphere or less. If there is a large amount of suspended material in the sample, filtration through the 0.45μ porosity filter pad should be preceded by filtration through an appropriate filter (for example, glass fiber) which is also pretreated as described above.

5.32 Autoclaving - should be used when it is desired to determine the amount of algal biomass that can be grown from all additional nutrients in the water, including those contained in filterable organisms, which can be solubilized by autoclaving. The sample should be autoclaved at 15 psi (1.1 kg-cm^2) at 250° F (121° C). The length of time of autoclaving will depend on the volume of the sample, e.g., 30 min or 10 min/l, whichever is longer. After autoclaving and cooling the sample should be allowed to equilibrate either in an air or carbon dioxide atmosphere in order to restore the carbon dioxide lost during autoclaving and to lower the pH to its original

level (it will generally rise on autoclaving). If an electronic particle counter is to be used for all counting, the carbon-dioxide equilibrated sample should then be passed through an 0.45μ membrane filter.

5.4 Storage - Although it is known that changes do occur in water samples during storage regardless of storage conditions, the extent or chemistry of these changes is not well defined, and therefore attempts should be made to minimize the duration of storage. Changes in samples should be minimized by keeping samples cool, in the dark, using proper containers, and avoiding air spaces over sample. Temporary storage prior to sample preparation should be in the dark at 0-4° C. If prolonged storage is anticipated, the sample should be prepared first and then stored in the dark at 0-4° C.

6. Synthetic Algal Nutrient Medium

6.1 Final concentration of nutrients.

6.11 Macronutrients - The following salts, Biological or Reagent grade, in milligrams per liter of glass-distilled water.

Compound	Concentration (mg/l)	Element	Concentration (mg/l)
$NaNO_3$	25.500	N	4.200
K_2HPO_4	1.044	P	0.186
$MgCl_2$	5.700	Mg	2.904
$MgSO_4 \cdot 7H_2O$	14.700	S	1.911
$CaCl_2 \cdot 2H_2O$	4.410	C	2.143
$NaHCO_3$	15.000	Ca	1.202
		Na	11.001
		K	0.469

6.12 Micronutrients - The following salts, Biological or Reagent grade, in micrograms per liter of glass-distilled water.

Compound	Concentration (µg/l)	Element	Concentration (µg/l)
H_3BO_3	185.520	B	32.460
$MnCl_2$	264.264	Mn	115.374
$ZnCl_2$	32.709	Zn	15.691
$CoCl_2$	0.780	Co	0.354
$CuCl_2$	0.009	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$FeCl_3$	96.000	Fe	33.051
$Na_2EDTA \cdot 2H_2O$	300.000		

6.2 Stock solutions.

6.21 Macronutrients - Stock solutions of individual salts may be made up in 1000 times the final concentration.

6.22 Micronutrients - The trace metals, $FeCl_3$ and EDTA are combined in a single stock mix at 1000 times final concentration.

6.3 Preparation of medium

6.31 Combination of stock solutions - 1 ml of each of the stock solutions (6.21 and 6.22) is added to glass-distilled water to give a final volume of 1 liter. The trace metal - $FeCl_3$EDTA mixture (6.12) is added after filtration.

6.32 Pretreatment of uninoculated reference medium - For some work, sterilization may not be required for experiments to be carried out with freshly prepared culture media since the recommended assay species are not axenic and therefore contain an assortment of symbiotic and

commensal (non-parasitic) bacteria. Stock cultures, however, should be maintained in previously sterilized culture medium. It is recommended that uninoculated sterile reference medium be stored in the dark to avoid any (unknown) photochemical changes.

6.33 Prolonged storage - Reference medium for stock cultures may be filter-sterilized as in 5.31 or autoclaved as suggested in 5.32.

7. Inoculum

7.1 Test algae (see Appendix 7.1 also).

7.11 Selenastrum capricornutum Printz.

7.12 Microcystis aeruginosa Kutz. emend Elenkin (Anacystis cyanea Drouet and Daily).

7.13 Anabaena flos-aquae (Lyngb.) De Brebisson.

7.14 Diatom - not yet selected. If one is used add 10 mg Si/l (101.214 mg $Na_2SiO_3 \cdot 9H_2O$/l) to the culture medium.

7.2 Source of test algae - Available from National Eutrophication Research Program, Pacific Northwest Water Laboratory, Environmental Protection Agency, 200 S.W. 35th Street, Corvallis, Oregon 97330.

7.3 Maintenance of stock cultures.

7.31 Medium - see 6.

7.32 Incubation conditions - 24 $\pm$ 2° C under continuous cool-white fluorescent lighting - 400 ft-c (4304 lux) $\pm$ 10 percent for S. capricornutum and 200 ft-c (2152 lux) $\pm$ 10 percent for M. aeruginosa and A. flos-aquae.

7.33 First stock transfer - Upon receipt of the inoculum species, a portion should be transferred to the algal culture medium in 6. (Example: 1 ml of inoculum in 100 ml of medium in 500 ml Erlenmeyer flask).

7.34 Subsequent stock transfers - A new stock transfer using an aseptic technique should be made as the first operation upon opening a stock culture. The volume of the transfers is not critical so long as enough cells are included to overcome significant growth lag. A routine stock transfer schedule, such as weekly, is recommended as a means of providing a continuing supply of "healthy" cells for experimental work.

7.35 Age of inoculum - Cultures, one to three weeks old, may be used as a source of inoculum. For Selenastrum a one-week incubation is often sufficient to provide enough cells. The blue-green species require a longer time to achieve maximum crop than does Selenastrum. This slower growth of the blue-green assay species should be considered in planning for sufficient inoculum to carry out required experimental work. Thus, two to three weeks may be required to provide inocula for assays with the blue-green species.

7.4 Preparation of inoculum - Cells from the stock culture should be centrifuged and the supernatant discarded. The sedimented cells should be resuspended in an appropriate volume of glass distilled water containing 15 mg $NaHCO_3$/l and again centrifuged. The sedimented algae should again be resuspended in the water-bicarbonate solution and used as the inoculum.

7.5 Amount of inoculum - The cells suspended in the bicarbonate solution (7.4) should be counted and pipetted into the test water to give a starting cell concentration in the test waters as follows:

S. capricornutum	10^3 cells/ml
M. aeruginosa	50×10^3 cells/ml
A. flos-aquae	50×10^3 cell/ml

The volume of the transfer is calculated to result in the above concentrations in the test flasks (Example: for S. capricornutum 5×10^5 cells/ml in the stock culture requires a 0.2 ml transfer per 100 ml of test water).

8. Test Conditions

8.1 Temperature. 24 ± 2° C.

8.2 Illumination - continuous "cool-white" fluorescent lighting - 400 ft-c (4304 lux) ± 10 percent for S. capricornutum and 200 ft-c (2152 lux) ± 10 percent for M. aeruginosa and A. flos-aquae. Intensity is measured adjacent to the flask at the liquid level (see Appendix 8.2).

9. Procedure

9.1 Preparation of glassware - The recommended procedure is as follows: All cylinders, flasks, bottles, centrifuge tubes and vials are washed with detergent or sodium carbonate and rinsed thoroughly with tap water. This is followed by a rinse with 10 percent solution by volume of reagent hydrochloric acid (HCl); vials and centrifuge tubes are filled with the 10 percent HCl solution and allowed to remain a few minutes, and all larger containers are filled to about one-tenth

capacity with HCl solution and swirled so that the entire inner surface is bathed. After the HCl rinse, the glassware is rinsed five times with tap water followed by five rinses with deionized water.

Pipettes are placed in 10 percent HCl solution for 12 hours or longer and then rinsed at least 10 times with tap water in an automatic pipette washer followed by a rinse with deionized water. Disposable pipettes may be used to eliminate the need for pipette washing and to minimize the possibility of contamination.

Cleaned glassware is dried at 105° C in an oven and is then stored either in closed cabinets or on open shelves with the tops covered with aluminum foil.

Before use, culture flasks are stoppered with plastic plugs or covered with aluminum foil and autoclaved at 15 psi for 15 minutes. Following autoclaving the flasks are prerinsed with the type medium to be used for subsequent culturing and placed for 20-30 minutes, inverted, on absorbant paper to drain.

9.2 pH Control - In order to insure the availability of carbon dioxide the pH should be maintained below 8.5. This can be accomplished by (1) using optimum surface to volume ratios; (2) by continuously shaking the flask (approximately 100 oscillations per minute); (3) by ventilation with air or air/carbon dioxide mixture; and, in extreme cases, by (4) bubbling air/carbon dioxide mixture through the culture.

9.3 Growth parameters - Two parameters are used to describe the growth of a test alga in the Bottle Test: maximum specific growth rate and maximum standing crop. Either or both of these parameters may be determined, depending on the objectives of any particular assay.

9.4 Maximum specific growth rate

9.41 The maximum specific growth rate (μ_{max}) for an individual flask is the largest specific growth rate (μ) occurring at any time during incubation. The μ_{max} for a set of replicate flasks is determined by averaging μ_{max} of the individual flasks.

The specific growth rate, μ, is defined by

$$\mu = \frac{\ln(X_2/X_1)}{t_2 - t_1} \quad \text{days}^{-1}$$

where X_2 = biomass concentration at end of selected time interval

X_1 = biomass concentration at beginning of selected time interval

t_2-t_1 = elapsed time (in days) between selected determinations of biomass

NOTE: If biomass (dry weight) is determined indirectly, e.g., by cell counts, the specific growth rate may be computed directly from these determinations without conversion to biomass, provided the factor relating the indirect determination to biomass remains constant for the time period considered.

9.42 Laboratory measurements - The maximum specific growth rate occurs during the logarithmic phase of growth--usually between day 0 and day 5--and therefore it is necessary that measurements of biomass be made at least daily during the first 5 days of incubation to determine this maximum rate. Indirect measurements of biomass, such as cell counts, will normally be required because of the difficulty in making accurate gravimetric measurements at low cell densities.

The time at which measurements are made should be recorded for use in the computations.

9.43 Computation of maximum specific growth rate - The maximum specific growth rate (μ_{max}) can be determined by calculation using the equation in Section 9.41 to determine the daily specific growth rate (μ) for each replicate flask and averaging the largest value for each flask (see Appendix 11.31). It may also be determined by preparing a semi-log plot of biomass concentration versus time for each replicate flask. Ideally, the exponential growth phase can be identified by 3 or 4 points which lie on a straight line on this plot. However, the data often deviate somewhat from a straight line, so a line judged to approximate most closely the exponential growth phase is drawn on the plot. If it appears that the data describe two straight lines, the line of steepest slope should be used. A linear regression analysis of the data may also be used to determine the best fit straight line. Two data points which most closely fit the line are selected and the specific growth rate (μ) is determined according to the equation given in Section 9.41 (see Appendix 9.43 and Appendix 11.31). The largest specific growth rates for the replicate flasks are averaged to obtain μ_{max}.

9.5 Maximum standing crop

9.51 Definition - The maximum standing crop in any flask is defined as the maximum algal biomass achieved during incubation. For practical purposes, it may be assumed that the maximum standing crop has been achieved when the increase in biomass is less than 5 percent per day.

9.62 Laboratory measurement - After the maximum standing crop has been achieved, the dry weight of algal biomass may be determined gravimetrically using either the aluminum-dish or filtration technique. For details of these techniques, see Appendix 9.61. If biomass is determined indirectly, the results should be converted to an equivalent dry weight using appropriate conversion factors.

9.6 Biomass monitoring - several methods may be used, but they must always be related to dry weight.

9.61 Dry weight - gravimetrically (see Appendix 9.61).

9.62 By direct microscopic counting (hemacytometer or plankton counting cell) or the use of an electronic particle counter. A flos-aquae, which is filamentous, is not amenable to counting with an electronic particle counter. Microscopic counting can be facilitated by breaking up the algal filaments with a high speed blender or by sonication (see Appendix 9.62).

9.63 Absorbance - with a spectrophotometer or colorimeter at a wavelength of 600-750 mμ. In reporting the results, the instrument make or model, the geometry and path length of the cuvette, the wave length used, and the equivalence to biomass should be reported (see Appendix 9.63).

9.64 Chlorophyll - after extraction or by direct fluorometric determination (see Appendix 9.64). The equivalence between chlorophyll content and biomass should be reported.

9.65 Total cell carbon - by carbon analyzer. Equivalence between total cell carbon and biomass should be reported.

10. Spikes

The quantity of cells that may be produced in a given medium is limited by the substance that is present in the lowest relative quantity with respect to the needs of the organism. If a quantity of the limiting substance were added to the medium, cell production would increase until this additional supply was depleted or until some other substance became limiting to the organism. Additions of substances other than that which is limiting would yield no increase in cell production. Nutrient additions may be made singly or in combination, including waste waters, and the growth response may be compared to unspiked controls to identify those substances which limit growth rate or cell production. The selection of spikes, e.g., nitrogen, phosphorus, iron, sewage effluents, etc., will depend on the answer being sought.

In all instances the volume of a spike should be as small as possible. The concentration of spikes will vary and must be matched to the waters being tested. Two considerations should be taken into account when selecting the concentration of spikes: (1) the concentration should be kept small to minimize alterations of the sample, but at the same time it should be sufficiently large to yield a potentially measurable response; and (2) the concentration of spikes should be related to the fertility of the sample. To assess the effect of nutrient additions, they must be compared to an unspiked control of the test water. If the control is quite fertile, cell production will be high and flask-to-flask variations in the controls might mask the effect of small additions of the limiting nutrient.

10.1 Auxiliary spikes - In addition to spikes for the purpose of determining stimulatory or inhibitory effects on algal growth in test waters, it is sometimes necessary to check for the possibility that the test water contains some toxic material which could influence results. To check for toxic materials, the test water may be spiked with the elements in complete synthetic medium (see Section 6). If no increase in growth occurs, the presence of toxic materials is suspected. In some situations, dilution of the sample or the addition of a chelator will eliminate toxic effects.

11. Data Analysis and Interpretation

11.1 Introduction - The fundamental measure used in the Algal Assay: Bottle Test to describe algal growth is the amount of suspended solids (dry weight) produced and determined gravimetrically. Other biomass indicators such as those shown in 9.6 may be used; however, all results presented must include experimentally determined conversion factors between the indicator used and the dry weight of suspended solids. Results should be expressed in the units shown in Appendix 9.6. Several different biomass indicators should be used whenever possible because biomass indicators may respond differently to any given nutrient limiting condition.

11.2 Reference Curves - Results of spiking assays should be presented together with the results from two types of reference samples; the assay reference medium and unspiked samples of the water under consideration. Preferably the entire growth curves should be presented for each of the two types of reference samples. The results of individual assays should be presented in the form of the maximum

specific growth rate (with time o occurrence) and maximum standing crop (with time at which it was reached).

11.3 Maximum specific growth rates - See Section 9.43.

11.31 Identification of growth rate limiting nutrients by single nutrient spikes - Growth rate limiting nutrients can be determined by spiking a number of replicate flasks with single nutrients, determining the maximum specific growth rate for each flask, and comparing the averages by a Student's t-Test, or other appropriate statistical tests (see Appendix 11.31).

11.32 Identification of growth rate limiting nutrients by spiking with many nutrients - Data analysis for multiple nutrient spiking can be performed by analysis of variance calculations. It is important in multiple nutrient spiking to account for the possible interaction between different nutrients; such interactions can readily be accounted for by means of the above mentioned factorial analysis. An example of an assay to determine the growth limiting nutrient is described in Appendix 11 together with a factorial analysis computer program.

11.4 Maximum standing crop - See Section 9.5.

11.41 Identification of growth limiting nutrient - The same methods which were described in Sections 11.31 and 11.32 for finding the growth rate limiting nutrient can be used to determine the nutrient limiting growth of the maximum standing crop (see Appendix 11.41).

11.42 Available concentration of growth limiting nutrient - The "Available Concentration" of the growth limiting nutrient can be determined by comparing the maximum standing crop in an unspiked sample with the maximum standing crop in the reference medium having varying concentrations of the growth limiting nutrient in question.

11.5 Confidence intervals - Both the maximum specific growth rate and the maximum standing crop should be presented with the confidence interval indicated. The calculation of confidence interval for the average values presented should be based on at least five samples. Consequently, a minimum of five replications should be made the first time when an unfamiliar source water is analyzed. The results of these five replicates are then used to calculate the standard deviation. Subsequent samples from the same source can be analyzed using only three replicates and reported with the confidence interval established for that source water (see Appendix 11.5).

11.6 Rejection of outliers - When algal assays are conducted it is often observed that one of the flasks among replicates shows a growth difference from the remainder of the replicates. Such outliers can be eliminated from the results if they fall outside the limits indicated in Appendix 11.6. A laboratory should keep track of individual flasks which result in outliers. Flasks which produce outliers more than once should not be used in further algal assays.

11.7 Evaluation of assay results - The overall evaluation of assay results consists of two parts. The first part is the determination whether a given assay result is significant when considered as a laboratory measurement. Several methods are available such as the Student's t and Analysis of Variance techniques presented in Appendix 11. However, it must be emphasized that there is as yet no unique method available to determine significant responses. Each experimental evaluation must be designed based upon the specific objectives using valid statistical procedures.

The second part of the overall evaluation is the correlation of laboratory assay results to effects observed or predicted in the field. This phase is underway but no specific guidelines are yet available; investigators should note the general considerations presented in Section 3 prior to the planning of any algal assays.

APPENDICES*

*Each Appendix is numbered to correspond with that Section to which its contents are related. Some Sections do not have a corresponding Appendix.

APPENDIX 3

3.2 Determination of limiting nutrients - This appendix is included to provide illustrations of how the technique of spiking may be used to conduct bioassays. The examples are included not necessarily to indicate how bioassays should be conducted, but rather to illustrate some of the rationale and logic that may be appropriate in certain instances. Depending upon the objectives, bioassays may be more elaborate or simpler than the examples given.

Example 1 - Spiking with One Nutrient

Assume that a sample has been collected from a relatively infertile lake, treated to remove indigenous organisms, and a bioassay is to be conducted to obtain answers to the following:

1. Which nutrient limits maximum cell production in the sample?

2. What is the biologically available concentration of that nutrient?

3. Is the sample sensitive to changes in the concentration of the limiting nutrient?

Assume further that preliminary testing has shown that nitrogen and phosphorus are the only two potentially limiting nutrients, and toxic materials are not present in the sample.

To achieve the stated objectives the following experimental design (Table 1) involving 57 flasks and 7 treatments can be used.

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TABLE 1

Experimental Design, Example 1

Treatment	No. Flasks
1. Lake Water Control	3
2. Phosphorus Spikes	
Lake Water + .005 mg P/l	3
Lake Water + .015 mg P/l	3
Lake Water + .050 mg P/l	3
3. Nitrogen Spikes	
Lake Water + .075 mg N/l	3
Lake Water + .225 mg N/l	3
Lake Water + .750 mg N/l	3
4. Combined Spikes	
Lake Water + .005 mg P/l + .075 mg N/l	3
Lake Water + .015 mg P/l + .225 mg N/l	3
Lake Water + .050 mg P/l + .750 mg N/l	3
5. Growth References - Phosphorus	
(Medium*-P)	3
(Medium -P) + .005 mg P/l	3
(Medium -P) + .015 mg P/l	3
(Medium -P) + .050 mg P/l	3
6. Growth References - Nitrogen	
(Medium*-N)	3
(Medium -N) + .075 mg N/l	3
(Medium -N) + .225 mg N/l	3
(Medium -N) + .750 mg N/l	3
7. Full-Strength Medium	3
Total	57

*Medium refers to the synthetic algal nutrient medium (see Section 6.).
(Medium -P) refers to the medium prepared without phosphorus.
(Medium -N) refers to the medium prepared without nitrogen.

1. Lake Water Control - Lake water blanks provide the basis for comparison of the other treatments and provide a measure of the general fertility of the sample. (In some instances, increased replication of the control would be desirable.)

2. Phosphorus Spikes - This series is included to determine if the sample is sensitive to additions of phosphorus. Since the sample was thought to be infertile, the concentrations of the spikes are relatively low. Three separate concentrations were selected to increase the possibility of obtaining a measurable response if, in fact, phosphorus was the limiting nutrient, and also to characterize the response of the sample to additions of phosphorus.

3. Nitrogen Spikes - Rationale same as for phosphorus spikes.

4. Combined Spikes - This series is included to investigate the possibility that both nitrogen and phosphorus were present in amounts so small that additions of these nutrients singly would not yield a measurable response.

5. Growth References - Phosphorus - The controls are included to: a) obtain a measure of "nutrient carry-over" in the inoculum (medium -P flasks); b) establish that the inoculum is quantitatively responsive to changes in the concentration of phosphorus; and c) provide a relative scale for quantifying the amount of phosphorus available to the test organism.

6. Growth References - Nitrogen - Rationale same as for phosphorus controls.

7. Full-Strength Medium - This treatment is included to provide a general check on cell growth and to provide an index for comparison to other assays.

3.3 Evaluation of Materials

Example (X)

Hypothetical Situation: Assume that a new household material will be introduced into a regional market, that the material will enter surface waters through the sewage system equipped with secondary treatment, that expected levels in the sewage will be 1 mg/l, and that there are two large lakes in the regional drainage basin. The lakes have in the past supported populations of green and blue-green algae and, therefore, Selenastrum capricornutum and Microcystis aeruginosa were selected as test organisms.

A test protocol for evaluating the effect of the new material on algae in the lake waters might be:

1. Blank Lake Water

New Material Spikes

2. Lake water + New Material at 0.1 mg/l
3. Lake water + New Material at 1.0 mg/l
4. Lake water + New Material at 10.0 mg/l

Sewage Spikes

*5. Lake water + primary effluent without New Material
*6. Lake water + primary effluent containing 10 mg/l New Material
7. Lake water + secondary effluent without New Material
8. Lake water + secondary effluent fed with primary effluent containing 10 mg/l of New Material

*Assuming a significant portion of primary effluent bypasses secondary treatment. Each variable is run in both lake waters. Sewages are added at a concentration of 1-2 percent by volume. Three replicates are run on each variable (3 reps. x 11 variables x 2 waters x 2 organisms = 132 flasks).

Nutrient Equivalent Spikes

9. Lake water + major nutrient (e.g., P) equivalent to that contained in 10 mg/l New Material

10. Lake water + major nutrient (e.g., N, etc.) equivalent to that contained in 10 mg/l New Material

11. Lake water + 10 percent medium* (as indicator of a possible toxicant present in lake water)

12. Medium - Optional (to make certain inoculum is viable)

3.4 Example of method to assess waste treatment processes - This example illustrates one possible application of the algal assay in the evaluation of the potential treatment process. The process evaluation described below was made on a process which consisted of percolation of a secondary effluent through approximately 400 ft of natural gravel.

The experimental design was based on local conditions where the process effluent would be discharged. In this case, the process effluent would be discharged directly into artificial lakes without any appreciable natural dilution. Consequently these tests were conducted on undiluted process effluent.

The maximum standing crop was chosen as the parameter to be evaluated based on the conditions of very long hydraulic residence times in the lakes which would receive the effluent. Selenastrum capricornutum Printz was selected as the test organism because unicellular green algae had been found to predominate in the lakes in previous years.

*Medium refers to synthetic algal culture medium (see Section 6).

The limiting nutrients were determined in two steps for both the influent to and effluent from the percolation grounds.

The first step consisted of dividing the possible growth limiting nutrients into four groups:

I Trace metals

II Iron and Manganese

III Phosphorous and Nitrogen

IV Macronutrients (Ca, Mg, Cl, etc)

These four groups were then added alone and in all possible combinations to samples of the influent and effluent and the maximum standing crop determined. Two replicates should be made for each of the combinations tested (i.e., a total of 32 tests for each water tested).

A typical factorial experiment layout is shown in Table 1. The results, cell counts, and dry weights can be analyzed as a 2x2x2x2 factorial experiment. A computer program for analyzing such a factorial experiment is shown in Appendix 11.7.

TABLE 1

DESIGN FOR 2^4 FACTORIAL EXPERIMENTS
TO DETERMINE LIMITING NUTRIENTS

FACTORS

30% * Macronutrients added	+								-							
30% * P & N added	+				-				+				-			
30% * Fe & Mn added	+		-		+		-		+		-		+		-	
30% * other trace metals added	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Rep I Flask No.	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31
Rep II Flask No.	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32
"Factor" in print out	15	13	14	10	12	9	7	4	11	8	6	3	5	2	1	

*30% of the reference medium (see 6.) concentration to both influent and effluent respectively

APPENDIX 5

5.3 Sample preparation - The ability of filtration and heat treatment techniques to remove "indigenous" algae from culture media as well as natural water samples enriched with algae was evaluated. After treatment, samples were incubated under standard Provisional Algal Assay Procedure batch conditions (without inoculation to detect growth of resistant algae). The change in chemical composition of the samples after treatment is shown in Table 1. Filtration through Whatman No. 41 filter paper removed up to 67 percent of the total phosphorus. Membrane filtration caused no apparent change in the COD or total phosphorus of an oligotrophic water but removed close to 67 percent of the total phosphorus of eutrophic water samples and from 22 to 71 percent of the Fe of samples. Centrifugation also removed substantial quantities (up to 55 percent) of the total phosphorus of samples.

Pasteurization, autoclaving and sonication obviously do not remove constituents from the water samples unless these are lost under the conditions used, e.g., volatile substances driven off or carbon dioxide evolution. However, total carbon and total phosphorus analyses of autoclaved and raw samples of eutrophic water showed no essential differences, although reductions of total phosphorus concentration in other eutrophic waters by autoclaving and pasteurization have been observed. These decreases were not consistently observed and were generally below 20 percent; however, in one instance the decrease was as high as 67 percent.

Analyses of Ca, Na, K, Mg, Cl, SO_4, and NO_3 showed that filtration and heat treatment did not significantly affect the concentrations of these ions as shown in Table 2. Small changes were observed but these could have been due to the precision of analyses. However, autoclaving appears to cause an increase in the soluble silica concentration, possibly from the glass containers.

TABLE 1

CHANGE OF COD AND TOTAL PHOSPHORUS CONTENT OF SAMPLES INDUCED BY FILTRATION AND CENTRIFUGATION TECHNIQUES

Pretreatment	Change in Concentration Range in Percent	
	COD	Total Phosphorus
Filtration (Whatman No. 41)	N.D.	5.0 to 66.9
Filtration (0.45μ to 0.5μ membranes)	0.0[a] to 72.3[b](4)	0.0[a] to 66.7[b]
Centrifugation	N.D.	13.8 to 54.9

[a]Oligotrophic water

[b]Eutrophic water

The effect of pretreatment on maximum standing crop and maximum specific growth rate is shown in Tables 3 and 4 respectively.

TABLE 2

EFFECT OF PRETREATMENT METHODS ON CHEMICAL CONSTITUENTS IN SAMPLES

Characteristic	Raw Water Nutrient Concentration C_o-mg/l	Pretreatment and Terminal Concentrations				
		F	A	FA	S	FS
		C_t-mg/l	C_t-mg/l	C_t-mg/l	C_t-mg/l	C_t-mg/l
SO_4	<10.0	<10.0	<10.0	<10.0	<10.0	<10.0
Ca	8.0	8.0	9.8	---	8.4	8.2
	5.1	5.1	---	---	---	---
	11.5	11.3	---	---	---	---
	9.6	10.4	---	---	---	---
	10.8	10.7	---	---	---	---
Na	6.1	6.6	6.4	6.6	6.3	6.8
K	0.7	0.6	0.6	0.6	0.6	0.6
Cl	4.0	5.0	4.0	4.0	4.0	5.0
NH_3	0.0	0.1	---	---	---	---
	0.0	0.0	---	---	---	---
	0.1	0.1	---	---	---	---
	0.6	1.2	---	---	---	---
NO_3	<.1	<.1	---	---	---	---
	<.1	<.1	---	---	---	---
	<.1	<.1	---	---	---	---
	<.1	<.1	---	---	---	---
Mg	.5	.4	---	---	---	---
	3.8	3.8	---	---	---	---
	5.4	5.8	---	---	---	---
	4.1	4.1	---	---	---	---
	---	3.2	---	3.2	---	3.3
Soluble Si	1.8	1.8	5.9	6.3	1.9	1.8

F = Filtered through 0.45 to 0.5μ pore size membranes
A = Autoclaved
S = Sonicated
FA = Filtered followed by autoclaving
FS = Filtered followed by sonication

TABLE 3

THE EFFECT OF PRETREATMENT ON THE MAXIMUM STANDING CROP

Relative Maximum Cell Production Response	Samples				
	Bantam Lake	Wawayanda Lake	Green Pond	Pond Water	Lake Anza
$\frac{\hat{X}_A}{\hat{X}_F}$	--	--	--	2.5	3.4
$\frac{\hat{X}_P}{\hat{X}_F}$	--	--	--	--	2.5
$\frac{\hat{X}_{FW}}{\hat{X}_F}$	--	--	--	--	3.1
$\frac{\hat{X}_A}{\hat{X}_{FW}}$	--	--	--	--	1.1
$\frac{\hat{X}_A}{\hat{X}_P}$	--	--	--	--	1.3
$\frac{\hat{X}_{FA}}{\hat{Y}_F}$	0.37 --	1.7 --	0.39 --	-- --	-- --
$\frac{\hat{X}_{PF}}{\hat{X}_F}$	-- 3.3	-- -	-- --	-- --	-- --
$\frac{\hat{X}_{AF}}{\hat{X}_F}$	15.7 17.7	5.6 --	0.5 --	-- --	-- --
$\frac{\hat{X}_{AF}}{\hat{X}_{PF}}$	-- 5.3	-- --	-- --	-- --	-- --
$\frac{\hat{X}_{AF}}{\hat{X}_{FA}}$	42.3 --	3.3 --	1.3 --	-- --	-- --

(Please refer to the following page for the key to Table 3.)

$\hat{X}$ maximum cell concentration	A autoclaving	FA filtration followed by autoclaving
- not determined	F filtration	PF pasteurization followed by filtration
	FW Whatman filtration	AF autoclaving followed by filtration
	P pasteurization	

TABLE 4

ALGAL GROWTH RATES FOR SEVERAL SAMPLE PRETREATMENTS

Treatment	Mean Maximum Specific Growth Rate Day^{-1}	Coefficient of Variation[b] %
Autoclaved	0.46[a]	26.4
Pasteurized	0.33	43.0
Filtration Whatman No. 41	0.25	24.0
Filtration Membrane 0.45 μ	0.25	29.3

[a] Mean growth rate of four replicate flasks.

[b] Coefficient of variation = standard deviation (100)/mean.

The maximum standing crop values of pasteurized samples were found to be significantly ($p \geq 0.95$) higher than the values of membrane filtered samples. Similar results were observed for Whatman No. 41 filtered and autoclaved samples when compared to membrane filtered samples. However, the results were quite variable and not statistically different (i.e., $p < 0.90$). Higher values were observed with heat treated samples as compared to membrane filtered samples as shown in Table 3.

Autoclaving of samples (whether followed by filtration or not) resulted in higher maximum standing crop values than observed with membrane filtered samples in all cases but one. The latter was an oligotrophic water in which membrane filtration apparently did not remove much particulate matter. Pasteurization also resulted in values higher than those for membrane filtration; however, generally the maximum standing crop of pasteurized samples was less than that of autoclaved samples. Membrane filtration reduced the maximum standing crop even more than that observed with Whatman No. 41 filtered samples. The highest values were obtained when samples were autoclaved, followed in order by pasteurization, Whatman filtration and membrane filtration.

Autoclaved samples resulted in the highest maximum specific growth rates with the pasteurized samples producing the next highest maximum specific growth rate. The lowest maximum specific growth rates were obtained in Whatman filtered and membrane filtered samples. The maximum specific growth rate of the autoclaved samples was observed to be significantly ($p \geq 0.95$) higher than the maximum specific growth rates of either filtration treatments.

APPENDIX 7

7.1 Comments on the Taxonomy, Morphology, and Reproduction of Algal Test Species.

It is recommended that three standard test organisms be used in the algal bioassay procedures for bottle tests and continuous flow chemostat tests, viz: Selenastrum capricornutum, Anabaena flos-aquae, and Microcystis aeruginosa. These three species are recommended for use because they provide a representative cross-section of the various different types of algal species likely to be found in a variety of waters of different nutritional status. Selenastrum is a unicellular or loosely aggregated colonial organism of the green algae or Chlorophyceae. The two remaining species are of the blue-green algae, or Cyanophyceae.

Anabaena flos-aquae is a filamentous organism in which heterocysts occur; it is, therefore, a species capable of fixing nitrogen; Microcystis is a unicellular or loosely-aggregated colonial organism in which heterocysts do not occur and it is, therefore, not a nitrogen fixer. The following comments on the structure, reproduction, and form of the three entities are presented for the benefit of those who wish to use the algal bioassay procedures but who are not fully acquainted with the test organisms.

7.11 Selenastrum capricornutum

Selanastrum capricornutum is a green alga, Chlorophyceae, of the order Chlorococcales. Entities attributed to this order are characterized by their unicellular or colonial habit in which the cells are in a non-motile condition either for their entire life history

or for the greater part of it. The systematic subdivision of the Chlorococcales is dependent upon the mode of reproduction, the cellular shape and characteristics of the colony. Selenastrum is characterized by the shape of individual cells which are curved in the shape of a new moon. These may occur as single cells or aggregated into groups, the cells being attached to one another at their convex faces by a relatively minute gelatinous mass. The cell or the aggregate of cells does not possess a prominent gelatinous envelope. The cell groups may contain 2 to 12 cells and several groups frequently become attached to one another to form cell masses containing 50 or more cells. The larger cell masses are not normally found in actively agitated cultures although they do occur frequently in field collections. There is usually a single chloroplast which in young cells lies on the convex side whereas in older cells it may appear to fill the cell completely. Some authors have reported a single pyrenoid, which is usually associated with starch formation in Chlorophyceae, whereas other authors have claimed that no pyrenoid is present. There is considerable variation in the size of individual cells, which may range from 10 to 48 μ in length and 3 to 9 μ in breadth. The cell size appears to reflect the state of nutrition and the rate at which cell division is occurring. Various species of the genus have been described on the basis of cell size although most of these would appear to be of very dubious validity The most frequent form of reproduction in Selenastrum capricornutum is by a process of cell division. Cells give rise directly to two or more cells of similar shape to the parent cell or colony; the daughter cells are usually referred to in the literature as "autospores."

7.12 Microcystis aeruginosa

Microcystis aeruginosa is a blue-green alga, Cyanophyceae, of the order Chroococcales. Entities attributed to this order are characterized by their unicellular or colonial habit and occurrence in mucilaginous masses which are commonly free-floating. The cells are small, spherical or ellipsoidal, 1 to 6 μ in diameter. As in all Cyanophyceae, there are no chloroplasts as such, the photosynthetic pigments being distributed generally throughout the cytoplasm of the cell giving it a granular appearance. The cells rarely occur singly but are usually aggregated into masses which are held together by a common gelatinous matrix of an extremely watery consistency. In quiet waters of ponds and lakes, these gelatinous masses may measure as much as a centimeter in diameter and be of a spherical, elongate or highly irregular shape. There are no heterocysts present and the cells do not appear to be differentiated from one another in any way. Reproduction appears to occur by division of the cells to give two daughter cells and the fragmentation of the gelatinous masses with age. There are no reports of any forms of special asexual or sexual reproduction in Microcystis.

7.13 Anabaena flos-aquae

Anabaena flos-aquae is a blue-green alga, Cyanophyceae, of the order Hormogonales. Entities attributed to this order are characterized by their unbranched filamentous organization in which heterocysts occur. The normal cells composing the filaments are spherical or slightly elongate in shape, 4 to 10 μ in length and 4 to 7 μ in breadth. The filament does not normally possess an obvious mucilaginous

sheath. As in all Cyanophyceae there are no chloroplasts as such, the photosynthetic pigments being distributed generally throughout the cytoplasm of the cell to give it a granular blue-green coloration. The filaments are usually straight or occasionally with a slight curvature but relatively stiff and rigid. Occasionally, the filaments may aggregate to form free-floating masses but this is extremely rare in culture. In quiet waters of ponds and lakes such masses may measure as much as a centimeter in diameter, floating on the surface as an obvious bloom. Heterocysts are cells which are much larger than ordinary vegetative cells and possess a well-developed thick wall. In Anabaena, the heterocysts normally occur only in an intercalary position. They measure 8 to 12 μ in length and 5 to 11 μ in breadth. The heterocysts are formed by the conversion of vegetative cells; this conversion involves the loss of the normal blue-green pigmentation and the development of a highly refractive cytoplasmic content of yellowish color. With age the cell contents of the heterocysts may disappear completely and they then appear as empty structures. The most crucial characteristic of the heterocysts is that they appear to be the sites of nitrogen fixation. Reproduction in Anabaena, as in most Cyanophyceae, is a process about which little is known. Vegetative fragmentation is probably the most frequent method of multiplication. In Anabaena, structures which are referred to in the literature as "spores" or "akinetes" occur with regularity. These are of an elongate cylindrical shape and much larger than the vegetative cells, measuring as much as 60 to 80 μ in length. They arise by the conversion of normal vegetative cells which increase markedly in size and develop a thickened wall. These structures may remain in a dormant condition in the field for a

considerable time before germinating to form a new filament. There are no indisputable reports of sexual reproduction in Anabaena, or other members of the Cyanophyceae for that matter.

APPENDIX 8

8.2 Illumination - This appendix describes the effect of light intensities on the three test organisms, Selenastrum capricornutum, Anabaena flos-aquae, and Microcystis aeruginosa. The data in Fig. 1 were obtained using the medium of the Provisional Algal Assay Procedure (PAAP), February 1969. Growth responses in the present medium (Sec. 6.) have not been tested under the same range of light intensities, but any differences due to a different medium are expected to be minor.

Plots of cell numbers show that the logarithmic growth phase occurs normally within the first week for all three species (Fig. 1). The growth rate of the green alga, Selenastrum, is almost double that of the blue-greens, Microcystis and Anabaena. Within the range of light intensities studied, the growth rate for all three species increased with increasing light intensity.

In culturing these species, therefore, producing enough cells to serve as a test inoculum may require a longer period of time for blue-greens than for greens, e.g., 2 weeks vs. 1 week, if a proposed test is of sufficient size to require a large number of cells.

The maximum standing crop, determined at the end of the three-week growth cycle as algal dry weight, also increased with increasing light intensity (Fig. 2). The relationship of maximum standing crop/light intensity was not so obvious, however, when the algal production was determined by cell counts (Fig. 1).

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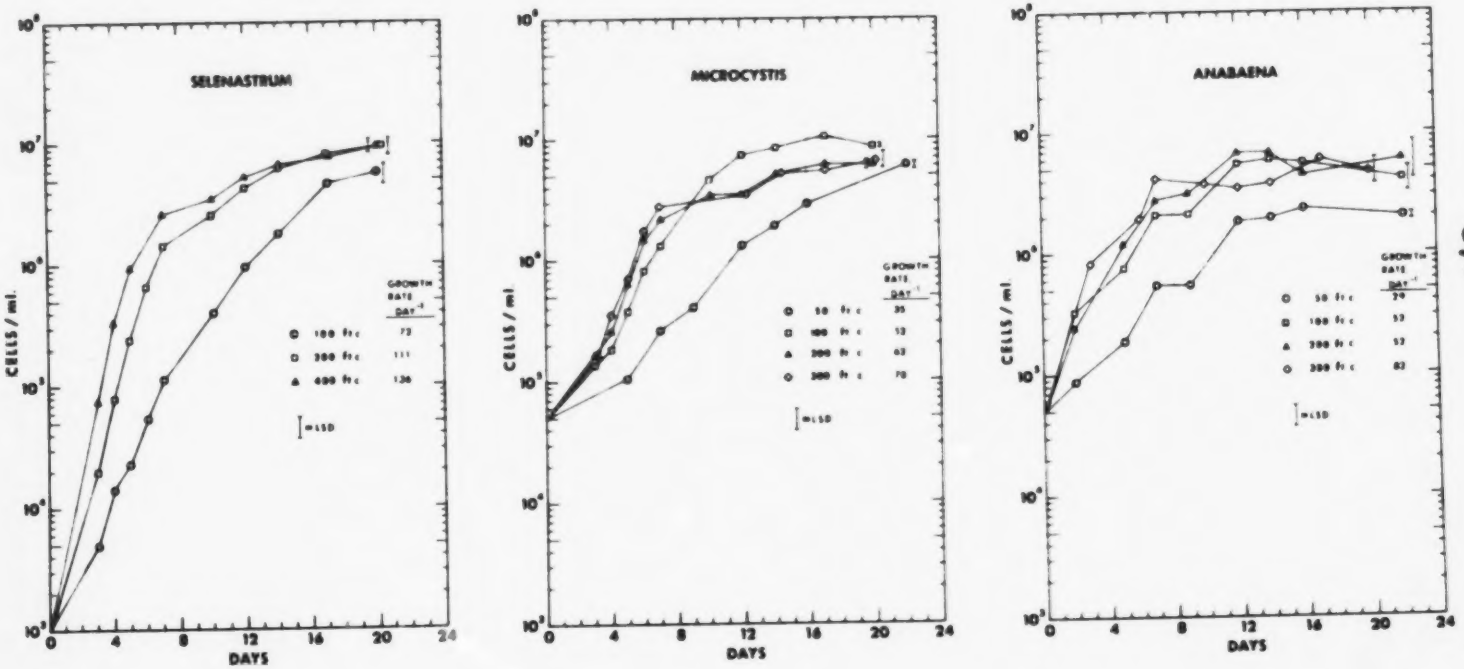


Figure 1. Effect of light intensity on the growth rate of the algal test species.

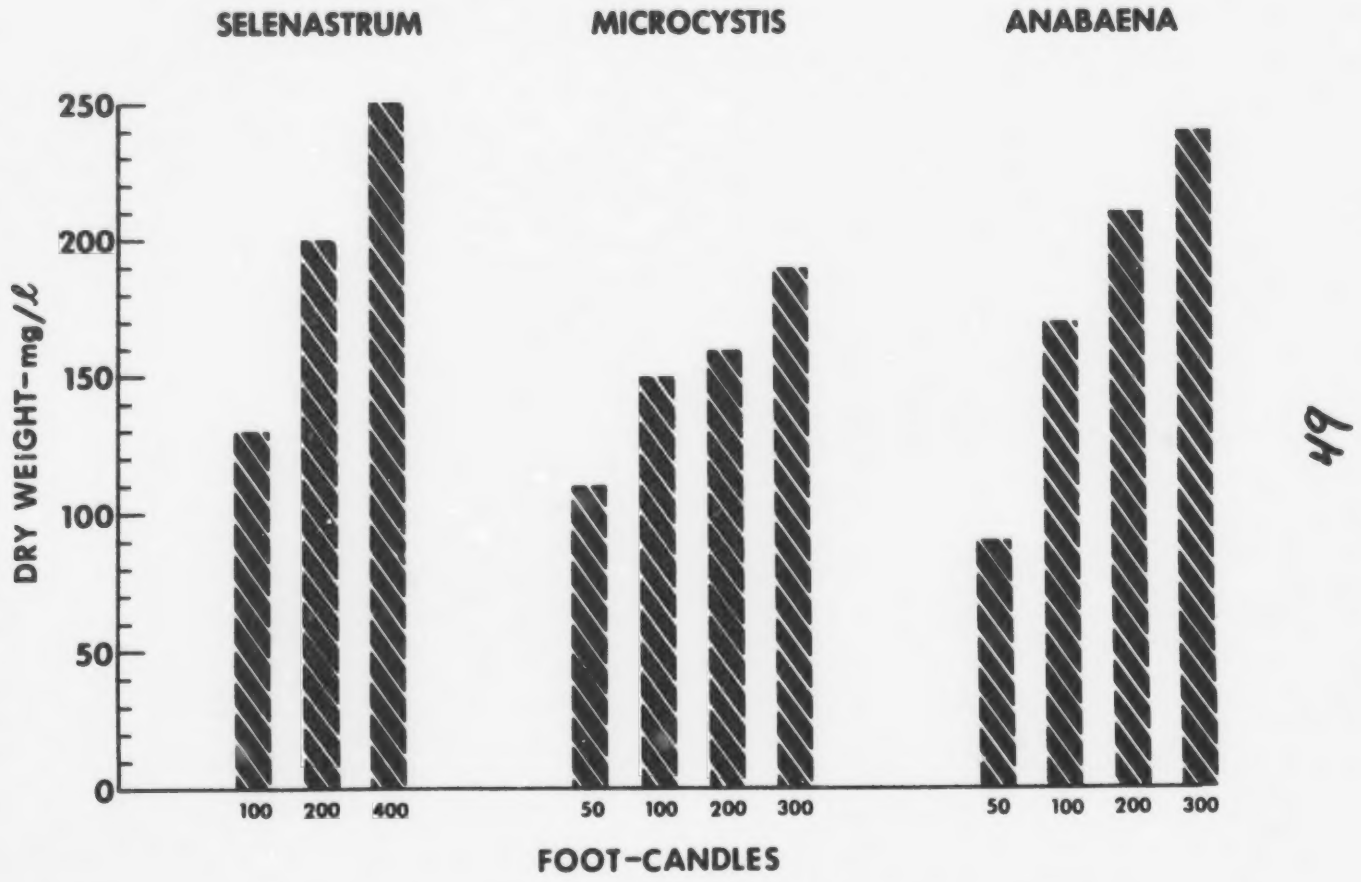


Figure 2. Effect of light intensity on the maximum standing crop of the algal test species.

In the aforementioned light intensity study, a conclusion was drawn that the three algal test species could be grown at one convenient light level of 200 ft-c. Nevertheless, as practically all the PAAP development work had been done with one organism, Selenastrum, at the light level of 400 ft-c., it was not seen fit to make a change. There also was evidence of a slight growth rate limitation of Selenastrum at 200 ft-c., and it was decided not to introduce that variable into the test. No data were collected at illumination levels above 400 ft-c. to determine if light at 400 ft-c. could be limiting for Selenastrum.

NOTE: Light meters used in the assay laboratory to determine light intensities should be standardized prior to use. Commercial laboratories can perform such standardizations by comparison with calibrated lamps supplied by the National Bureau of Standards.

Standardization should include both intensity and color temperature comparisons if the light meter to be used is designed for a color temperature different from the color correlated temperature (4200°K) of the "Cool-White" fluorescent light source specified in the assay procedure.

APPENDIX 9

9.43 Computation of maximum specific growth rates (see graph on next page).

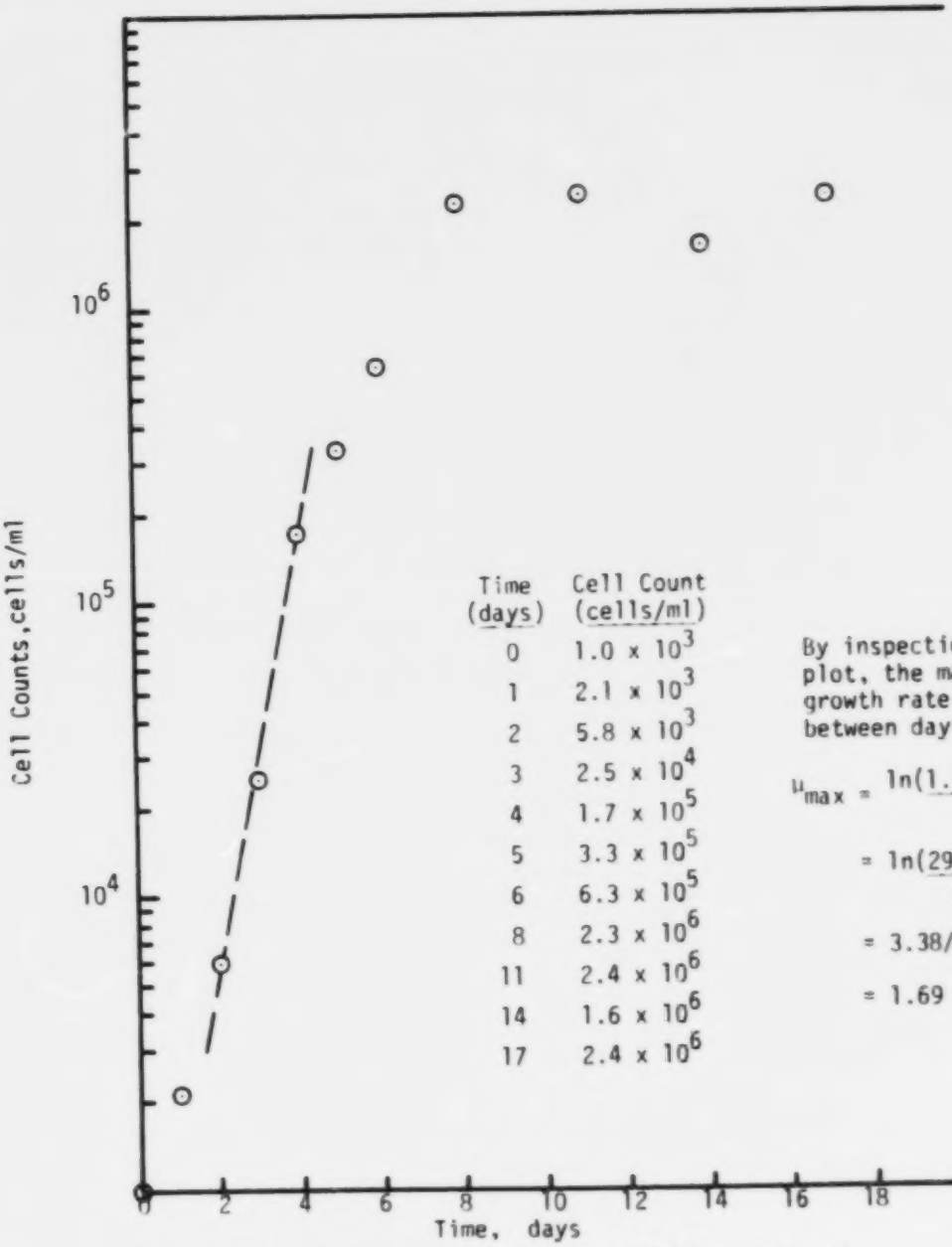
9.6 Biomass monitoring

MEASUREMENT	UNITS
Suspended solids	$mg \cdot l^{-1}$
Absorbance (optical density)	$units \cdot cm^{-1}$
Suspended carbon	$mg \cdot l^{-1}$
Cell counts	$number \cdot ml^{-1}$
Cell volume*	$\mu^3 \cdot ml^{-1}$
Chlorophyll fluorescence	$units \cdot cm^{-1}$
Extracted chlorophyll	$\mu g \cdot l^{-1}$

9.61 Dry weight - This method will be particularly useful for assessing the growth of Anabaena flos-aquae. The cells of this alga grow in filaments and it is difficult to obtain accurate cell counts. The method may also be used with S. capricornutum, M. aeruginosa, and other species of algae. In any case, however, it should only be used with either relatively dense cultures or large volumes of thinner cultures. Otherwise, the error may be large. Two methods may be employed.

Method I -- A suitable portion of algal suspension is centrifuged, the sedimented cells washed three times in distilled water containing 15 mg $NaHCO_3$/l, transferred to tared crucibles or aluminum cups, dried overnight in a hot air oven at 105°C and weighed. This

*Mean cell volume (μ^3/cell) times cell count (cells/ml).



Time (days)	Cell Count (cells/ml)
0	1.0×10^3
1	2.1×10^3
2	5.8×10^3
3	2.5×10^4
4	1.7×10^5
5	3.3×10^5
6	6.3×10^5
8	2.3×10^6
11	2.4×10^6
14	1.6×10^6
17	2.4×10^6

By inspection of the plot, the maximum growth rate occurred between days 2 and 4.

$$\mu_{max} = \frac{\ln(1.7\times10^5/5.8\times10^3)}{2}$$

$$= \ln\frac{(29.3)}{2}$$

$$= 3.38/2$$

$$= 1.69 \text{ days}^{-1}$$

Figure 1. Computation of maximum specific growth rate.

method is more sensitive than Method II, but is open to error through loss of cells during washing.

Method II -- This method involves filtering a measured portion of algal suspension through a tared Millipore(R) filter. The filter recommended is type AA with an 0.80 micron pore size.

The method is as follows:

1. Dry filters for several hours at 90°C in an oven. The filters may be placed in folded sheets of paper upon which the weights or codes may be written.

2. Cool filters in a desiccator containing desiccant.

3. Filter a suitable measured aliquot of the culture under a vacuum of 0.5 atmosphere. Normally 10 ml is sufficient, but in thin cultures more may be required.

4. Rinse the filter funnel with 50 ml distilled water using a wash bottle and allow the rinsings to pass through the filter. This serves to transfer all of the algae to the filter and to wash the nutrient salts from the filter.

5. Dry the filter in its paper folder at 90°C, cool in desiccator, and weigh.

6. To correct for loss of weight of filters during washing, wash two blank filters with 50 ml of distilled water, pouring it through slowly under reduced vacuum. Dry and weigh filters and record weight loss. This correction is not large, but is essential for meaningful results on thin cultures. For example, if 10 ml have been filtered and yield a difference between tare and final of 1.10 mg and the blank has lost 0.02 mg, then the culture contains:

$$(1.10 + 0.02) \times 100 = 112 \text{ mg/l dry weight}$$

NOTE: a. The drying temperature has been selected to avoid damage to filters.

b. In weighing, do not hang the filters over the edge of the pan or electrostatic attraction of the filter to the balance will result in an error.

9.62 By direct microscopic counting - Apparent irregularities in the Anabaena growth curves are often due to the problem inherent in counting filamentous algae, i.e., the difficulty in breaking up the filaments properly, without cell damage, in order to obtain a representative sample for counting. Filament-breaking techniques which have been used with varying degrees of success include the use of (1) a syringe, (2) an ultrasonic bath, (3) a high speed blender, and (4) vigorous swirling with glass beads. While none of these techniques is without drawbacks, expelling the sample forcefully through a syringe against the inside of the flask is the most satisfactory way to break up tight clumps of filaments. Other methods of biomass measurement such as dry weight, absorbance, or chlorophyll fluorescence are often considered to be more suitable than cell counts for growth assessment of filamentous algae.

9.63 Absorbance - Absorbance or optical density, as defined by Beer's Law Expression $D = \log \frac{1}{I} = \log \frac{I_0}{I_x} = \alpha c l$ is usually derived for absorption of light by molecules of solute in homogenous solution. It can be derived also for a suspension of uniform particles but with some necessary added restrictions. For particles of bacterial or algal cell size I_x is less than I_0 by virtue of absorption and also

by virtue of scattering caused by cell reflections and refractions. The fraction of the latter which reach the light measuring receiver depends upon the instrument design. A large receiver close to the cuvette catches much of the scattered light (i.e., is insensitive to scattering). A small receiver far from the cuvette in a long-focused or diaphragmed optical path catches very little scattered light (i.e., is very sensitive to scattering. Reference: Mastre, H., J. Bacteriology, 30:335 (1935).

Most current instruments are likely to be more sensitive to scattering than to absorption as evidenced by effect of wavelength. A simple test is the following: For a green alga, light absorption by pigments *in vivo* will show relative optical densities for 600: 680: 750 mμ ratios, such as 70: 500: 1 (ratios probably correct in order of magnitude). In practical measurements, without elaborate precautions to avoid effects of scattering, the ratios will always be very much less.

In any photometric measure of optical density or absorbance considerations of precision lead to simple rule of thumb that measurements be limited to a range of $0.05 < D < 1.0$. A further reason for this restriction is that for particulate suspensions the linearity between D and concentration holds practically only over limited range. Hence, values measured should be limited to this range by concentration or dilution before measurement.

A common and necessary check upon instrumentation is to measure D on various dilutions of an algal suspension. By this means optical density (D) can be calibrated against any other measure of cell quantity (X) such as cell number or dry weight. There is no

assurance that the relation between D and X will be constant and independent of culture conditions. As noted above, the optical density measured is a complex function of volume, size and pigmentation of the cells. Hence, the relation between D and X should be examined on different batches of algae which best simulate actual conditions of the test.

9.64 Chlorophyll - The use of fluorescence to determine phytoplankton chlorophyll (extracted and in vivo) has been reported to be more sensitive and less cumbersome than the familiar trichometric method (Yentsch and Menzel, 1963; Lorenzen, 1966).

Chlorophyll, like many organic molecules, possesses the ability to fluoresce. Simply stated, fluorescence is the ability of a substance to absorb light energy at one wavelength and emit this energy at a longer wavelength. Yentsch (1963) reported maximum absorption (excitation) of chlorophyll in an acetone solution at 430 mμ and maximum emission between 650 and 675 mμ.

Lorenzen (1966) reported that the fluorescence of in vivo chlorophyll is considerably less efficient than dissolved chlorophyll, yielding only about 1/10 as much fluorescence per unit weight as the same amount in solution.

Strickland and Parsons (1965) state that many plant cells resist complete extraction in acetone and certain species retain 50-90 percent of their pigments. The use of a sonic disintegrator may have limited value in increasing extraction efficiency. The use of a tissue grinder, as recommended by Yentsch and Menzel (1963), is relatively convenient and improves results on many natural populations,

but even this approach fails to give complete extraction in a reasonable time with certain species.

The correlation of (in vivo) fluorescence units and cells per milliliter of Selenastrum capricornutum (Figure 1) shows excellent agreement during the logarithmic phase of growth, indicating the usefulness of fluorescence to evaluate productivity. It should be pointed out that the chlorophyll a content of algal cells grown in culture medium, natural waters, and sewage effluents is not always constant and is subject to change dependent upon the physiological condition of the cells. Nitrogen deficient cells, for example, usually have a lower chlorophyll a content than non-nitrogen limited cells even though the cell mass (cells/ml and dry weight) may be in close agreement. However, in vivo (direct) fluorescence measurements can aid in the evaluation of (valid) increases in cell counts attributed to increased algal growth.

The following methods of fluorometric determination of chlorophyll a in vivo (directly) and after solvent extraction are recommended.

I. Equipment and Supplies.

Millipore filtration equipment designed to hold 47 mm diameter membrane or glass filters. Stoppered graduated centrifuge tubes of 15-ml capacity having both glass and polyethylene stoppers and "small volume" cuvettes having a cell path of 1.0 cm, but holding 10 ml or less of solution.

Fluorometer capable of measuring chlorophyll, e.g., modified Turner or equivalent. Tissue grinder. Arthur H. Thomas #4288-B or equivalent laboratory stirring motor.

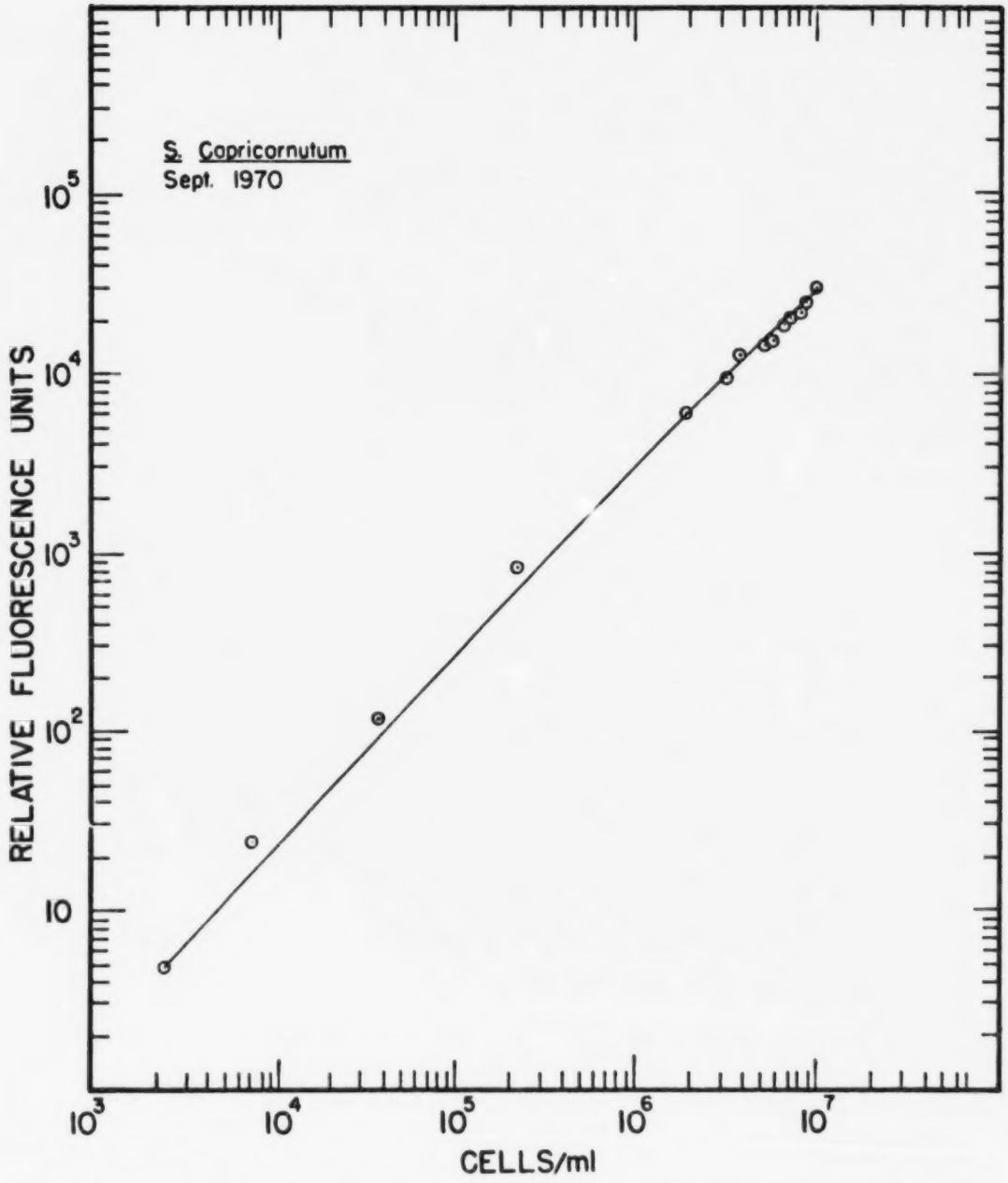


Figure 1. Relationship of Cell Numbers to Relative Fluorescence Units

II. Reagents.

A. 90 Percent acetone: Distill reagent grade acetone over about 1 percent of its weight each of anhydrous sodium carbonate and anhydrous sodium sulphite. Collect fraction boiling at a constant temperature near 56.5°C (uncorrected). Pipette 100 ml of distilled water into a one-liter volumetric flask and bring up to mark with acetone to contain exactly 1000 ml. Store tightly stoppered in a dark glass bottle between use. This reagent can be conveniently dispensed from a polyethylene wash bottle which should be kept nearly full.

B. Magnesium carbonate suspension. Add approximately 1 gram of finely powdered magnesium carbonate (light wt. or "Levis" grade) of analytical reagent quality to 100 ml of distilled water in a stoppered Erlenmeyer flask. Shake vigorously to suspend powder immediately before use.

III. In vivo (direct reading) procedure.

A. Sampling

1. Swirl flasks to insure homogenous suspension of algal cells.

2. Pipette cell suspension aliquot (5 ml minimum) into small beaker or vial.

B. Measure Fluorescence

1. Zero fluorometer with a distilled water blank before each sample reading and change in sensitivity setting.

2. Pour well-mixed sample into cuvette and read fluorescence. If reading (scale deflection) is over 90 units, use lower sensitivity setting, e.g., 30 x >10 x >3 x >1; conversely, if reading is less than 15 units, increase sensitivity setting.

3. If samples fail to stay in range, dilute accordingly.

4. Record fluorescence units, based on a common sensitivity factor, e.g., a reading of 50 @ 1 x = 1500 @ 30 x.

IV. Extraction (dissolved chlorophyll) procedure.

A. Filter measured sample under vacuum through a glass fiber filter. Add ≈ 1.0 ml magnesium carbonate suspension and drain filter thoroughly under suction.

B. Place filter into the bottom of tissue grinding tube. Add 2 ml of 90 percent acetone to the grinding tube and insert pestle.

C. Grind the sample 1-2 minutes (in subdued light) and wash pestle and grinding tube with 5 ml of 90 percent acetone into a 15 ml screw cap centrifuge tube. Centrifuge (2000 x g) for 1-5 min., allow to stand in the dark for 1-2 hrs to ensure the complete removal of all extractable pigments.

D. Measure fluorescence as outlined in step B direct reading procedure. If phaeophytin is to be measured, acidify with 2 drops 1.N HCl and reread fluorescence.

E. Fluorescence values can be recorded as relative chlorophyll values or as chlorophyll a (milligrams per cubic meter) as calculated from the equation:

$$\text{chlorophyll } a \text{ } (\text{mg m}^{-3}) = \frac{\frac{Fo/Fa_{max}}{(Fo/Fa_{max}) - 1} (kx) (Fo - Fa)}{\text{liters filtered}}$$

$$\text{phaeophytin a } (\text{mg m}^{-3}) = \frac{\frac{Fo/Fa_{max}}{(Fo/Fa_{max}) - 1} (kx) [Fo/Fa_{max}(Fa) - Fo]}{\text{liters filtered}}$$

where: Fo = fluorescence before acidification

Fa = fluorescence after acidification

Fo/Fa_{max} = maximum acid factor which can be expected in the absence of phaeophytin

kx = calibration constant for a specific sensitivity scale.

It should be noted that kx, Fo/Fa_{max}, and acid ratios are functions of the combination of photomultiplier and color filters.

References.

Lorenzen, C. J. 1966. A method for the continuous measurement of in vivo chlorophyll concentration. Deep-Sea Res. 13,223-227.

Strickland, J. D. H. and T. R. Parsons. 1965. A manual of sea water analysis. Fisheries Research Board of Canada, Bulletin No. 125, 2nd Revised Edition, 203 p.

Yentsch, C. S. and D. W. Menzel. 1963. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep-Sea Res. 10, 221-231.

APPENDIX 11

11.2 Reference curves - The results of an assay where cell counts were used as the biomass indicator are presented in Table 1. Triplicate sets of flasks were incubated for the control (lake water sample), the control with a phosphorus addition, and the control with a nitrogen addition. Figure 1 shows the average cell counts obtained in the same triplicate sets of flasks plotted against time (days).

11.3 Maximum specific growth rate

11.31 Identification of growth rate limiting nutrients by single nutrient spikes. The data shown in Table 1 were obtained by single nutrient spikes of nitrogen and phosphorus to a lake water sample. The daily specific growth rates (μ) for each flask of each triplicate set of flasks were calculated from the equation in Section 9.41 and are shown in Table 2. The largest daily specific growth rate for each flask is its μ_{max} and these were averaged to obtain μ_{max} for each set of triplicate flasks (Table 3). The phosphorus-spiked set of flasks had a μ_{max} of 1.33 ± 0.12, as opposed to a μ_{max} of 0.91 ± 0.16 in the nitrogen-spiked flasks and of 0.71 ± 0.14 in the control flasks. Phosphorus, therefore, can be identified as the growth rate limiting nutrient.

11.4 Maximum standing crop - The maximum standing crop can be identified from the data contained in Table 4 where the daily cell counts (Table 1) were averaged. The daily average cell count in the control, for example, increased steadily for the first four days. After that the daily increase was less than five percent and the maximum standing crop was determined to be 7.5×10^3 cells/ml.

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TABLE 1

TYPICAL REPORT OF ASSAY RESULTS

Time (Days)	CELLS PER ML								
	CONTROL			CONTROL + 0.05 mg P/l			CONTROL + 0.37 mg N/l		
	1	2	3	1	2	3	1	2	3
0	$1.0x10^3$	$1.0x10^3$	$1.0x10^3$	$1.0x10^3$	$1.0x10^3$	$1.0x10^3$	$1.0x10^3$	$1.0x10^3$	$1.0x10^3$
1	$2.1x10^3$	$1.7x10^3$	$1.6x10^3$	$3.3x10^3$	$2.8x10^3$	$3.1x10^3$	$2.2x10^3$	$1.9x10^3$	$2.3x10^3$
2	$4.1x10^3$	$3.6x10^3$	$3.0x10^3$	$9.3x10^3$	$8.1x10^3$	$8.2x10^3$	$5.9x10^3$	$4.7x10^3$	$4.4x10^3$
3	$5.5x10^3$	$4.4x10^3$	$4.8x10^3$	$3.4x10^4$	$3.3x10^4$	$3.0x10^4$	$6.2x10^3$	$5.1x10^3$	$6.1x10^3$
4	$7.8x10^3$	$8.1x10^3$	$6.6x10^3$	$1.1x10^5$	$1.1x10^5$	$1.1x10^5$	$6.5x10^3$	$7.8x10^3$	$7.1x10^3$
5	$7.9x10^3$	$8.2x10^3$	$6.7x10^3$	$1.3x10^5$	$1.5x10^5$	$1.4x10^5$	$7.6x10^3$	$8.5x10^3$	$8.3x10^3$
6	$8.2x10^3$	$8.2x10^3$	$6.8x10^3$	$1.5x10^5$	$1.6x10^5$	$1.6x10^5$	$9.0x10^3$	$9.7x10^3$	$9.6x10^3$
7	$8.4x10^3$	$7.2x10^3$	$6.9x10^3$	$1.6x10^5$	$1.6x10^5$	$1.6x10^5$	$9.0x10^3$	$9.9x10^3$	$9.8x10^3$

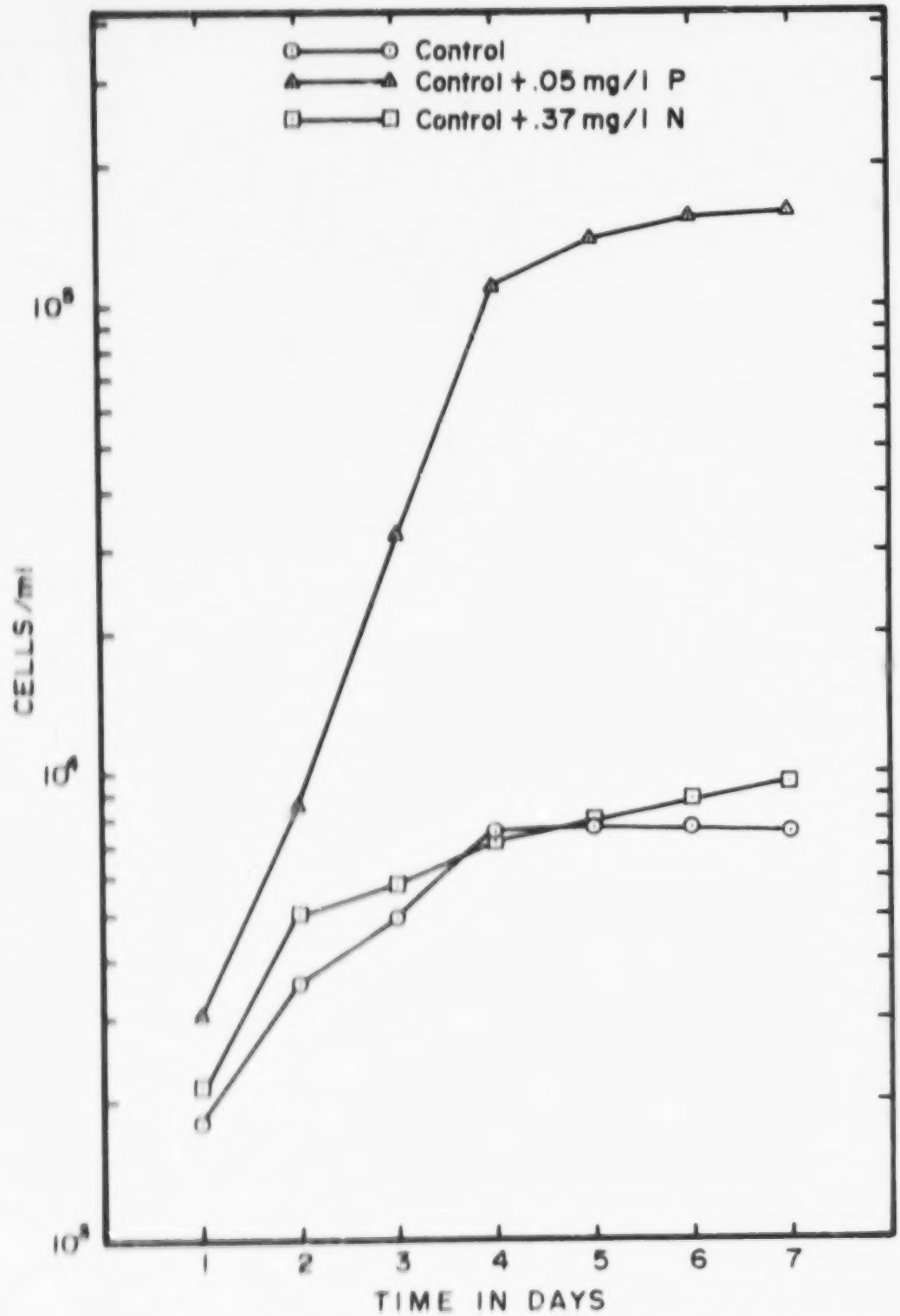

Figure 1. Effect of the addition of nitrogen and phosphorus to a lake water on the growth of Selenastrum capricornutum.

TABLE 2

TYPICAL REPORT OF GROWTH RATE CALCULATIONS

Time (Days)	SPECIFIC GROWTH RATES (μ) - Day^{-1}								
	CONTROL			CONTROL + 0.05 mg P/1			CONTROL + 0.37 mg N/1		
	1	2	3	1	2	3	1	2	3
0	0.74	0.53	0.47	1.19	1.03	1.13	0.79	0.64	0.83
1	0.67	0.75	0.63	1.04	1.06	0.97	0.98	0.91	0.65
2	0.29	0.20	0.47	1.29	1.40	1.30	0.05	0.08	0.33
3	0.35	0.61	0.32	1.17	1.20	1.30	0.05	0.42	0.15
4	0.01	0.01	0.02	0.17	0.31	0.24	0.16	0.09	0.16
5	0.04	0.00	0.01	0.14	0.06	0.13	0.17	0.13	0.15
6	0.02	0.00	0.01	0.06	0.00	0.00	0.00	0.02	0.02
7									

TABLE 3

DETERMINATION OF MAXIMUM SPECIFIC GROWTH RATES

Flask Number	Maximum Specific Growth Rate (μ_{max})		
	Control	Control + 0.05 mg P/l	Control + 0.37 mg N/l
1	0.74	1.29	0.98
2	0.75	1.40	0.91
3	0.63	1.30	0.83
Ave (μ_{max})	0.71 ±0.14	1.33 ±0.12	0.91 ±0.16

11.41 Identification of growth limiting nutrients - The data in Table 4 can be used to identify the growth limiting nutrient. The maximum standing crop for the control (7.5×10^3 cells/ml) is compared to that for the nitrogen-spiked flasks (9.43×10^3 cells/ml) and the phosphorus-spiked flasks (1.57×10^5 cells/ml). Since the phosphorus added to the lake water resulted in a significant increase in the maximum standing crop, it can be identified as the growth limiting nutrient.

11.5 Confidence intervals - Confidence intervals are based upon the standard deviation (σ).

σ = confidence interval of 66.6 percent

2σ = confidence interval of 95.0 percent

3σ = confidence interval of 99.0 percent

$$\sigma = \pm \sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/n}{n - 1}}$$

Example of calculation - Taking data (cell counts) from Table 1 for the triplicate set of control flasks for day one, the constants in the equation are as follows:

$x_1 = 2.1 \times 10^3$ $\quad x_1^2 = 4.41 \times 10^3$

$x_2 = 1.7 \times 10^3$ $\quad x_2^2 = 2.89 \times 10^3$

$x_3 = 1.6 \times 10^3$ $\quad x_3^2 = 2.59 \times 10^3$

$n = 3$

TABLE 4

CALCULATION OF MAXIMUM STANDING CROP

Time (Days)	CONTROL	CONTROL + 0.05 mg P/l	CONTROL + 0.37 mg N/l
	Ave. cells/ml 2σ	Ave. cells/ml 2σ	Ave. cells/ml 2σ
0	$1.00 \times 10^3 \pm 0.00 \times 10^3$	$1.00 \times 10^3 \pm 0.00 \times 10^3$	$1.00 \times 10^3 \pm 0.00 \times 10^3$
1	$1.80 \times 10^3 \pm 0.52 \times 10^3$	$3.07 \times 10^3 \pm 0.50 \times 10^3$	$2.13 \times 10^3 \pm 0.42 \times 10^3$
2	$3.57 \times 10^3 \pm 1.10 \times 10^3$	$8.53 \times 10^3 \pm 1.33 \times 10^3$	$5.00 \times 10^3 \pm 1.58 \times 10^3$
3	$4.90 \times 10^3 \pm 1.12 \times 10^3$	$3.23 \times 10^4 \pm 0.42 \times 10^4$	$5.80 \times 10^3 \pm 1.22 \times 10^3$
4	$7.50 \times 10^3 \pm 1.58 \times 10^3$	$1.10 \times 10^5 \pm 0.00 \times 10^5$	$7.13 \times 10^3 \pm 1.30 \times 10^3$
5	$7.60 \times 10^3 \pm 1.58 \times 10^3$	$1.40 \times 10^5 \pm 0.20 \times 10^5$	$8.13 \times 10^3 \pm 0.94 \times 10^3$
6	$7.73 \times 10^3 \pm 1.60 \times 10^3$	$1.57 \times 10^5 \pm 0.12 \times 10^5$	$9.43 \times 10^3 \pm 0.76 \times 10^3$
7	$7.50 \times 10^3 \pm 1.58 \times 10^3$	$1.60 \times 10^5 \pm 0.00 \times 10^5$	$9.57 \times 10^3 \pm 0.98 \times 10^3$

2σ = Standard Deviation at 95 percent confidence interval.

Maximum Standing Crop

Control = $7.50 \times 10^3 \pm 1.58 \times 10^3$ (Day 4)

Control + 0.05 mg P/l = $1.57 \times 10^5 \pm 0.12 \times 10^5$ (Day 6)

Control + 0.37 mg N/l = $9.43 \times 10^3 \pm 0.76 \times 10^3$ (Day 6)

Therefore: $\Sigma x^2 = 9.86 \times 10^3$

$$(\Sigma x)^2 = (5.4 \times 10^3)^2 = 29.16 \times 10^3$$

$$\frac{(\Sigma x)^2}{n} = \frac{29.16 \times 10^3}{3} = 9.72 \times 10^3$$

$$\sigma = \pm \sqrt{\frac{9.86 \times 10^3 - 9.72 \times 10^3}{2}} = \pm \sqrt{0.07} = \pm 0.26$$

$$2\sigma = \pm 0.52$$

$$3\sigma = \pm 0.78$$

Following is an example of determining the required number of replicates:

Consider the design of an experiment to compare two media, one of known strength which will produce a maximum standing crop of about $m_1 = 2 \times 10^6$ cells/ml and another medium expected to produce a greater standing crop. The "null hypothesis," which one expects to disprove, is that $m_2 \leq m_1$, i.e., that the unknown medium produces a standing crop not larger than the known medium. The "alternative hypothesis," which one expects to accept, is that $m_2 > m_1$, i.e., that the unknown medium produces a greater standing crop than the known medium.

How many replicate pairs of flasks should be used? The answer can be found by first answering the following five questions and then consulting the tables which follow.

Question 1: "What significance level, α, should be used?" For this example we shall use the significance level $\alpha = 0.05$, i.e., if the two media are the same strength ($m_1 = m_2$) there will be one chance in twenty that the experiment will result in the erroneous conclusion that the known medium is weaker ($m_1 < m_2$).

Question 2: "What is the smallest difference, $\delta = m_2 - m_1$ which must be detected?" The known medium will produce a standing crop of about $m_1 = 2 \times 10^6$ cells/ml. Suppose the other medium must produce a 10 percent greater crop ($m_2 = 2.2 \times 10^6$ cells/ml) to be "significantly" stronger, i.e., the smallest difference which must be detected is about $\delta = m_2 - m_1 = 2.0 \times 10^6 - 2.2 \times 10^6 = 2 \times 10^5$ cells/ml.

Question 3: "With what probability must a difference of δ_0 ($= 2 \times 10^5$ cells/ml) be detected by the experiment?" Suppose it is desired to have a probability of detection of 0.90, i.e., if the true difference in the standing crops of the media is 2×10^5 cells/ml there is a 90 percent chance the experiment will detect the difference (lead to the conclusion that the known medium is weaker). Conversely, there is a 10 percent chance the experiment will fail to detect a difference of 2×10^5 cells/ml. Denote the probability of detection as $1 - \beta = 0.90$.

Question 4: "What is the standard deviation, s, of an individual observation?" (Note that this is not the same as the standard error of a mean of several observations.) There would probably be some information about the standard deviation from a prior experiment with the "known" medium. For this example assume that previous experience indicates a standard deviation of approximately 2.7×10^5 cells/ml.

Question 5: "Does the alternative hypothesis specify a 'one-tail' alternative ($\delta > 0$, $m_2 > m_1$) or a 'two-tail' alternative ($\delta \neq 0$,

$m_2 \neq m_1$)?" In this example it is assumed the findings will be significant only if the unknown medium produces a greater standing crop than the known medium; thus the alternative hypothesis specifies a one-tail alternative ($\delta > 0$, $m_2 > m_1$). Therefore, a one-tail test will be used. (A two tail alternative would require a two-tail test.)

In summary, the answers to the questions above have provided the following values:

(1) $\alpha = 0.05$ = significance level

(2) $\delta_0 = 2 \times 10^5$ cells/ml = smallest "significant" difference

(3) $1 - \beta = 0.90$ = probability of detecting smallest significant difference

(4) $s = 2.7 \times 10^5$ cells/ml = standard deviation

(5) Alternative hypothesis specifies a one-tail test.

We can now compute the value of "d" and find the required number of replicates from the table:

$$d = 0.7071\ \delta_0/s$$
$$= (0.7071) \times (2 \times 10^5)/(2.7 \times 10^5)$$
$$= 0.524$$

Entering the One-Tail Test Tables with these values we find the number of replicates should be between 54 + 1 (corresponding to d = 0.4) and 24 + 1 (corresponding to d = 0.6)*. One should use quadratic interpolation in the table, but linear interpolation produces an approximate result: 36 replicate pairs. Note that only 10 replicate pairs would have the desired probability of detecting the difference if d = 1.0, i.e., if

*Note that the tabled value is not the number of replicates; one must add 1 to the tabled values in the $\alpha = .05$ table and 2 to the tabled values in the $\alpha = 0.01$ table.

TABLE 5

AID IN COMPUTING SAMPLE SIZES REQUIRED TO DETECT PRESCRIBED DIFFERENCES BETWEEN AVERAGES

Notation:

α Significance level of the test

δ_0 Smallest detectable or significant difference

$1-\beta$ Probability of declaring $\delta \neq 0$ if $\delta = \delta_0$

s Sample estimate of the standard deviation of an observation

d $= 0.7071\ \delta_0 / s$

ONE TAIL TEST TABLES:

For $\alpha = .01$ add 2 to the tabled value to get the number of pairs (replicates); for $\alpha = .05$ add 1 to the tabled value to get the number of pairs.

$\alpha = .01$

d \ $1-\beta$	.50	.60	.70	.80	.90	.95	.99
.1	542	666	813	1004	1302	1578	2165
.2	136	167	204	251	326	395	542
.4	34	42	51	63	82	99	136
.6	16	19	23	28	37	44	61
.8	9	11	13	16	21	25	34
1.0	6	7	9	11	14	16	22
1.2	4	5	6	7	10	11	16
1.4	3	4	5	6	7	9	12
1.6	3	3	4	4	6	7	9
1.8	2	3	3	4	5	5	7
2.0	2	2	3	3	4	4	6
3.0	1	1	1	2	2	2	3

$\alpha = .05$

d \ $1-\beta$	.50	.60	.70	.80	.90	.95	.99
.1	271	361	471	619	857	1083	1578
.2	68	91	118	155	215	271	395
.4	17	23	30	39	54	68	99
.6	8	11	14	18	24	31	44
.8	5	6	8	10	14	17	25
1.0	3	4	5	7	9	11	16
1.2	2	3	4	5	6	8	11
1.4	2	2	3	4	5	6	9
1.6	2	2	2	3	4	5	7
1.8	1	2	2	2	3	4	5
2.0	1	1	2	2	3	3	4
3.0	1	1	1	1	1	2	2

TABLE 5 (continued)

TWO TAIL TEST TABLES:

For α = .01 add 2 to the tabled value to get the number of pairs (replicates); for α = .05 add 1 to the tabled value to get the number of pairs.

α = .01

d \ 1-β	.50	.60	.70	.80	.90	.95	.99
.1	664	801	962	1168	1488	1782	2404
.2	166	201	241	292	372	446	601
.4	42	51	61	73	93	112	151
.6	19	23	27	33	42	50	67
.8	11	13	16	19	24	28	38
1.0	7	9	10	12	15	18	25
1.2	5	6	7	9	11	13	17
1.4	4	5	5	6	8	10	13
1.6	3	4	4	5	6	7	10
1.8	3	3	3	4	5	6	8
2.0	2	3	3	3	4	5	7
3.0	1	1	2	2	2	2	3

If we must estimate σ from our sample and use Student's t, then we should add 4 to the tabulated values to obtain the approximate required sample size. (If we are comparing two product averages, add 2 to the tabulated values, to obtain the required size of each sample. For this case, we must have $\sigma_A = \sigma_B$.)

α = .05

d \ 1-β	.50	.60	.70	.80	.90	.95	.99
.1	385	490	618	785	1051	1300	1838
.2	97	123	155	197	283	325	460
.4	25	31	39	50	66	82	115
.6	11	14	18	22	30	37	52
.8	7	8	10	13	17	21	29
1.0	4	5	7	8	11	13	19
1.2	3	4	5	6	8	10	13
1.4	2	3	4	5	6	7	10
1.6	2	2	3	4	5	6	8
1.8	2	2	2	3	4	5	6
2.0	1	2	2	2	3	4	5
3.0	1	1	1	1	2	2	3

$u = (z_{1-\alpha}+z_{1-\beta})^2 / d^2$, where z_x denotes the cumulative distribution function of the standard normal (0,1) distribution.

Source: Experimental Statistics, by Mary G. Natrella, National Bureau of Standards Handbook 91, U.S. Government Printing Office, Washington, D.C. The tables above are Tables A-9 and A-8, respectively, from this reference.

$\delta_0 = \frac{ds}{0.7071} = \frac{(1.0)(2.7 \times 10^5)}{0.7071} = 3.81 \times 10^5$. That is, 10 replicate pairs would have a 90 percent chance of detecting a difference of 3.8×10^5 cells, a 19 percent increase in standing crop, whereas 36 replicates are required to ensure a 90 percent chance of detecting a 10 percent increase in standing crop. These figures assume the validity of the estimate of the standard deviation.

11.6 Rejection of outliers - An "outlier" among replicate observations is one whose deviation from the mean is far greater than the rest in absolute value and perhaps lies three or four standard deviations or further from the mean. The outlier is a peculiarity and indicates a data point which is not at all typical of the rest of the data. It follows that an outlier should be submitted to particularly careful examination to see if the reason for its peculiarity can be determined.

Rules have been proposed for rejecting outliers, i.e., for deciding to remove the observation(s) from the data, after which the data are reanalyzed with these observations. Automatic rejection of outliers is not always a wise procedure. Sometimes the outlier is providing information which other data points cannot due to the fact that it arises from an unusual combination of circumstances which may be of vital interest and requires further investigation rather than rejection. As a general rule outliers should not be rejected out of hand unless they can be traced to causes such as errors in recording observations or in setting up apparatus. Otherwise careful investigation is in order. (The above was adopted from section 3.8 of Applied Regression Analysis by N. R. Draper and H. Smith, John Wiley and Sons, 1968.)

The following test may be applied for rejecting outliers:

1. Rank order the data in the group containing the outliers (all observations in the group are supposedly treated alike):

$$x_1 \leq x_2 \leq \cdots x_n$$

2. Compute the appropriate criterion:

If x_1 is the outlier $c = \frac{x_2 - x_1}{x_n - x_1}$

If x_n is the outlier $c = \frac{x_n - x_{n-1}}{x_n - x_1}$

3. If c exceeds the critical value opposite "n" in the following table, reject the outlier.

n	Critical Values	
	$\alpha = 0.05$	$\alpha = 0.01$
3	0.941	0.988
4	0.765	0.889
5	0.642	0.780
6	0.560	0.698
7	0.507	0.637

Example - Suppose the following replicate observations were made: 9.8, 4.7, 8.4, 8.0, 8.4, and 7.9. The value 4.7 is suspected to be an outlier. Rank order the data.

x_1	x_2	x_3	x_4	x_5	x_6	$n = 6$
4.7	7.9	8.0	8.4	8.4	9.8	

The criterion is as follows:

$$c = \frac{x_2 - x_1}{x_n - x_1} = \frac{7.9 - 4.7}{9.8 - 4.7} = \frac{3.2}{5.1} = 0.63$$

Since n = 6, this value is significant at the $\alpha = 0.05$ level ($0.63 > 0.560$), but not at the $\alpha = 0.01$ level ($0.63 < 0.698$).

The experimenter who is willing to discard five percent of all his good data would discard the observation 4.7 as an outlier. The experimenter who is willing to discard only one percent of all his good data would keep the observation unless he can determine an experimental reason for rejecting it.

4. If there are two suspected outliers (say x_1 and x_n or x_1 and x_2, the test may be repeated; apply it to the "worst" outlier first.

5. Note that the regular use of this procedure will result in discarding five percent (if $\alpha = 0.05$) or one percent (if $\alpha = 0.01$) of all one's good (valid) observations.

11.7 Evaluation of assay results - A computer program in BASIC, which can analyze a 2x2x2x2 FACTORIAL experiment, is presented in Table 6. Typical results of analysis of variance for such a growth rate experiment are shown in Tables 7, 8, and 9 for an influent to and effluent from a ground percolation process. In this example none of the nutrients or groups of nutrients limit the maximum specific growth rate (μ_{max}) of the test algae in the influent (Table 8). In the effluent (Table 9), both Factor 3 (P + N) and Factor 4 (macronutrients) were significant at the 95 and 99 percent confidence levels respectively.

The evaluation of significance, however, should always include a comparison of absolute effects. In this case a comparison of Mean 1 (with spiking) with Mean 2 (without spiking) shows that, although the spiking with macronutrients was statistically significant at a confidence level greater than 99 percent, the absolute increase in μ_{max} due to spiking was less than 10 percent.

TABLE 6

PROGRAM FOR ANALYSIS OF 2x2x2x2
FACTORIAL EXPERIMENTS

Prepared in BASIC for a HP2116B Computer

```
LIST
1  REM  ANALYSIS OF VARIANCE PROGRAM  K. JUSTICE.  SCHOOL OF
5  REM  ENGINEERING UNIV. OF CALIF.  IRVINE,CALIF. 92664
10  REM THIS PROGRAM WILL PERFORM AN ANALYSIS OF VARIANCE AND
15  REM COMPUTE F-RATIOS FOR A 2X2X2X2 (2↑4) FACTORIAL EXPERIMENT
20  REM WITH TWO REPLICATES.  TO USE, TYPE:
25  REM "200 DATA X(1,1),X(1,2),X(2,1),X(2,2).....X(M,N)"
30  REM WHERE M IS THE NUMBER OF SAMPLES AND N IS THE NUMBER
35  REM OF REPLICATES.
40  REM USE AS MANY 'DATA STATEMENTS' AS NECESSARY, NUMBERING
45  REM THEM SUCCESSIVELY.
100  DATA 16,2
300  DIM U[17,3],T[17],R[17]
305  READ M,N
309  FOR I=0 TO 16
310  LET T[I+1]=R[I+1]=0
312  NEXT I
315  LET G=R2=R=W=0
330  FOR I=1 TO M
335  FOR J=1 TO N
340  READ U[I+1,J+1]
345  LET T[I+1]=T[I+1]+U[I+1,J+1]
350  LET R=R+U[I+1,J+1]↑2
355  NEXT J
360  LET G=G+T[I+1]
365  LET W=W+T[I+1]↑2
370  NEXT I
375  LET A=(W-(G↑2)/M)/(N*(M-1))
380  LET B=(R-W/N)/(M*(N-1))
411  FOR J=1 TO N
412  FOR I=1 TO M
413  LET R[J]=R[J]+U[I+1,J+1]
414  NEXT I
415  LET R2=R2+R[J]↑2
416  NEXT J
417  LET R3=R2/M-G↑2/(M*N)
418  LET E1=B*M*(N-1)-R3
419  LET E=E1/(M-1)
420  LET E1=B*M*(N-1)-R3
421  LET E=E1/(M-1)
422  LET R3=R3/(N-1)
424  PRINT "              A N A L Y S I S   O F   V A R I A N C E"
425  PRINT "                  ";M;"SAMPLES OF SIZE ";N
435  PRINT "              SAMPLES","SAMPLE TOTAL","SAMPLE MEAN"
445  FOR I=1 TO M
450  PRINT "             "2*I-1;2*I,T[I+1],T[I+1]/N
455  NEXT I
460  PRINT
465  PRINT "          MEAN SQUARE (BETWEEN SAMPLES) = ";A
470  PRINT "          MEAN SQUARE (WITHIN SAMPLES)  = ";B
497  PRINT "          MEAN SQUARE (REPLICATES)      = ";R3
498  PRINT "          ERROR                         = ";E
499  PRINT
```

```
500  GOTO 820
700  DATA 1,-1,1,-1,1,-1,1,-1,1,-1,1,-1,1,-1,1,-1
705  DATA 1,1,-1,-1,1,1,-1,-1,1,1,-1,-1,1,1,-1,-1
710  DATA 1,1,1,1,-1,-1,-1,-1,1,1,1,1,-1,-1,-1,-1
715  DATA 1,1,1,1,1,1,1,1,-1,-1,-1,-1,-1,-1,-1,-1
720  DATA 1,-1,-1,1,1,-1,-1,1,1,-1,-1,1,1,-1,-1,1
725  DATA 1,-1,1,-1,-1,1,-1,1,1,-1,1,-1,-1,1,-1,1
730  DATA 1,-1,1,-1,1,-1,1,-1,-1,1,-1,1,-1,1,-1,1
735  DATA 1,1,-1,-1,-1,-1,1,1,1,1,-1,-1,-1,-1,1,1
740  DATA 1,1,-1,-1,1,1,-1,-1,-1,-1,1,1,-1,-1,1,1
745  DATA 1,1,1,1,-1,-1,-1,-1,-1,-1,-1,-1,1,1,1,1
750  DATA 1,-1,-1,1,-1,1,1,-1,1,-1,-1,1,-1,1,1,-1
755  DATA 1,-1,-1,1,1,-1,1,-1,-1,1,1,-1,-1,1,1,-1
760  DATA 1,1,-1,-1,-1,1,1,-1,1,-1,1,1,1,-1,-1,-1
765  DATA 1,-1,1,-1,-1,1,-1,1,-1,1,-1,1,1,-1,1,-1
770  DATA 1,-1,-1,1,-1,1,1,-1,-1,1,1,-1,1,-1,-1,1
820  DIM K[16,15]
830  FOR J=1 TO 15
835  FOR I=1 TO 16
840  READ K[I,J]
845  NEXT I
850  NEXT J
875  PRINT "      FACTORIAL ANALYSIS WITH FOUR TREATMENTS AT TWO LEVELS"
877  PRINT
885  PRINT "     FACTOR     COMPARISON      F-RATIO        MEAN 1";
886  PRINT "          MEAN 2"
895  FOR J=1 TO 15
910  LET S=Z1=Z2=0
920  FOR I=1 TO 16
930  LET C=C+K[I,J]*T[I+1]
931  IF K[I,J]=0 THEN 934
932  LET Z1=Z1+T[I+1]
933  GOTO 940
934  LET Z2=Z2+T[I+1]
940  NEXT I
950  LET S=(C*2)/(16*M)
960  LET F=S/E
990  PRINT USING "#,#,F,Z1/16,Z2/16
1005 NEXT J
1006 PRINT
1007 PRINT "        F = 4.54     * SIGNIFICANT AT 95% LEVEL"
1008 PRINT "        F = 8.68     ** SIGNIFICANT AT 99% LEVEL"
1020 END
```

TABLE 7

COMPUTER PRINTOUT CODE

FACTOR: Refers to the elements added as shown in code above. Each factor line in printout refers to a statistical comparison between those flasks which had the elements added versus those flasks which did not have the same elements added.

COMPARISON: Sign indicates direction of effect of addition. A negative sign means that addition of that particular combination of factors resulted in a decrease.

F-RATIO: * Indicates significance at the 95 percent level.
**Indicates significance at the 99 percent level.

MEAN: 1 Indicates mean of flasks with elements added.
2 Indicates mean of flasks without elements added.

FACTOR	ELEMENTS ADDED (1/3 MEDIUM CONCENTRATION)
1	(Trace)
2	(Fe + Mn)
3	(P + N)
4	(Macro)
5	(Trace) (Fe + Mn)
6	(Trace) (P + N)
7	(Trace) (Macro)
8	(Fe + Mn) (P + N)
9	(Fe + Mn) (Macro)
10	(P + N) (Macro)
11	(Trace) (Fe + Mn) (P + N)
12	(Trace) (Fe + Mn) (Macro)
13	(Fe + Mn) (P + N) (Macro)
14	(Trace) (P + N) (Macro)
15	(Trace) (Fe + Mn) (P + N) (Macro)

TABLE 8

INFLUENT TO PERCOLATION PROCESS

Maximum Growth Rate (Day^{-1})

```
READY
RUN

                A N A L Y S I S   O F   V A R I A N C E
                    16     SAMPLES OF SIZE  2
 SAMPLES                    SAMPLE TOTAL     SAMPLE MEAN
 1       2                   2.907            1.4535
 3       4                   3.072            1.536
 5       6                   2.956            1.478
 7       8                   3.001            1.5005
 9       10                  3.087            1.5435
 11      12                  3.17             1.585
 13      14                  2.941            1.4705
 15      16                  2.988            1.494
 17      18                  2.959            1.4795
 19      20                  3.041            1.5205
 21      22                  3.135            1.5675
 23      24                  3.022            1.511
 25      26                  3.062            1.531
 27      28                  2.905            1.4525
 29      30                  3.014            1.507
 31      32                  2.86             1.43

MEAN SQUARE (BETWEEN SAMPLES) =  3.65499E-03
MEAN SQUARE (WITHIN SAMPLES)  =  7.08389E-03
MEAN SQUARE (REPLICATES)      =  1.08795E-02
ERROR                         =  6.83085E-03

FACTORIAL ANALYSIS WITH FOUR TREATMENTS AT TWO LEVELS

FACTOR     COMPARISON          F-RATIO          MEAN 1          MEAN 2
  1      2.00033E-03          1.83054E-05      1.50281         1.50369
  2      .286002              .374207          1.51269         1.49481
  3      6.60033E-02          1.99300E-02      1.50581         1.50169
  4      .124002              7.03449E-02      1.50763         1.49988
  5     -.348                 .554031          1.49288         1.51463
  6     -.36                  .592899          1.4925          1.515
  7     -.681999              2.12786          1.48244         1.52506
  8     -.556001              1.41425          1.48638         1.52113
  9      .413999              .784104          1.51669         1.49081
 10     -.566001              1.46558          1.48606         1.52144
 11     -.282001              .363812          1.49494         1.51256
 12      3.59998E-02          5.92891E-03      1.50488         1.50263
 13     -5.60002E-02          1.43468E-02      1.502           1.5055
 14      .2                   .182994          1.51            1.4975
 15      .114                 5.94544E-02      1.50731         1.50019

F > 4.54 :  * SIGNIFICANT AT 95% LEVEL
F > 8.68 : ** SIGNIFICANT AT 99% LEVEL

READY
```

TABLE 9

EFFLUENT FROM PERCOLATION PROCESS

Maximum Growth Rate (Day^{-1})

RUN

A N A L Y S I S O F V A R I A N C E

16 SAMPLES OF SIZE 2

SAMPLES		SAMPLE TOTAL	SAMPLE MEAN
1	2	3.424	1.712
3	4	3.509	1.7545
5	6	3.425	1.7125
7	8	3.625	1.8125
9	10	3.409	1.7045
11	12	3.527	1.7635
13	14	3.467	1.7335
15	16	3.352	1.676
17	18	3.181	1.5905
19	20	3.339	1.6695
21	22	3.199	1.5995
23	24	3.409	1.7045
25	26	3.222	1.611
27	28	3.06	1.53
29	30	3.2	1.6
31	32	3.153	1.5765

MEAN SQUARE (BETWEEN SAMPLES) = 1.25570E-02
MEAN SQUARE (WITHIN SAMPLES) = 3.24726E-03
MEAN SQUARE (REPLICATES) = 2.07520E-03
ERROR = 3.32540E-03

FACTORIAL ANALYSIS WITH FOUR TREATMENTS AT TWO LEVELS

FACTOR	COMPARISON	F-RATIO	MEAN 1	MEAN 2
1	-.447	1.87768	1.65794	1.68588
2	-.159	.237576	1.66694	1.67688
3	.721003	* 4.88517	1.69444	1.64938
4	1.975	** 36.6556	1.73363	1.61019
5	4.89998E-02	2.25629E-02	1.67344	1.67038
6	-.859	* 6.93414	1.64506	1.69875
7	-.123001	.156383	1.66788	1.67594
8	-.250998	.592037	1.66406	1.67975
9	.159	.237576	1.67688	1.66694
10	-.264999	.659927	1.66363	1.68019
11	.284999	.763295	1.68081	1.663
12	-.285	.763301	1.663	1.68081
13	-.217	.442513	1.66513	1.67869
14	.295	.817806	1.68113	1.66269
15	.411	1.58741	1.68475	1.65906

F > 4.54 : * SIGNIFICANT AT 95% LEVEL
F > 8.68 : ** SIGNIFICANT AT 99% LEVEL

READY

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